ORIGINAL ARTICLE

Metabolic abnormalities in Williams–Beuren syndrome

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ABSTRACT

Background Williams–Beuren syndrome (WBS, OMIM-194050) is a neurodevelopmental disorder with multisystemic manifestations caused by a 1.55–1.83 Mb deletion at 7q11.23 including 26–28 genes. Reported endocrine and metabolic abnormalities include transient hypercalcaemia of infancy, subclinical hypothyroidism in ~30% of children and impaired glucose tolerance in ~75% of adult individuals. The purpose of this study was to further study metabolic alterations in patients with WBS, as well as in several mouse models, to establish potential candidate genes.

Methods We analysed several metabolic parameters in a cohort of 154 individuals with WBS (data available from 69 to 151 cases per parameter), as well as in several mouse models with complete and partial deletions of the orthologous WBS locus, and searched for causative genes and potential modifiers.

Results Triglyceride plasma levels were significantly decreased in individuals with WBS while cholesterol levels were slightly decreased compared with controls. Hyperbilirubinemia, mostly unconjugated, was found in 18.3% of WBS cases and correlated with subclinical hypothyroidism and hypotriglyceridemia, suggesting common pathogenic mechanisms. Haploinsufficiency at *MLXIPL* and increased penetrance for hypomorphic alleles at the *UGT1A1* gene promoter might underlie the lipid and bilirubin alterations. Other disturbances included increased protein and iron levels, as well as the known subclinical hypothyroidism and glucose intolerance.

Conclusions Our results show that several unreported biochemical alterations, related to haploinsufficiency for specific genes at 7q11.23, are relatively common in WBS. The early diagnosis, follow-up and management of these metabolic disturbances could prevent long-term complications in this disorder.

INTRODUCTION

Williams–Beuren syndrome (WBS, OMIM 194050) is a neurodevelopmental disorder characterised by multisystemic manifestations of variable expressivity, including dysmorphic features, supravalvular aortic stenosis and other vascular stenosis, hyperacusis and intellectual deficit with an uneven neurocognitive profile presenting relative strengths in language and weaknesses in visuospatial construction.[1] It has an incidence of 1/7500–1/20 000 births.[2 3] WBS is caused by a recurrent 1.55–1.83 Mb heterozygous deletion at chromosome band 7q11.23, which includes 26–28 genes.[4]

Several metabolic and endocrine abnormalities have been reported in patients with WBS. Transient hypercalcaemia has been documented in approximately 15% of infants and children, although further studies are needed to determine its exact prevalence at all ages.[5] Hypercalcaemia is usually mild and may be accompanied by hypercalciuria in some cases.[3] A more frequent metabolic abnormality is subclinical hypothyroidism. Studies focused on thyroid function and morphology have shown that approximately 25% of children with WBS present a thyroid-stimulating hormone (TSH) elevation and 70% present mild thyroid hypoplasia.[6 7] Finally, it has been reported that up to 75% of adult individuals with WBS develop diabetes or a prediabetic state of impaired glucose tolerance after the administration of a standard oral glucose tolerance test.[8]

Different approaches have provided insight into the contribution of the deleted genes in the WBS critical region to the metabolic phenotype observed in patients with WBS.[2 3] Clinical–molecular correlations in patients with partial deletions suggested that glucose intolerance might be caused by haploinsufficiency at genes of the centromeric half of the deletion interval, the gene coding for MLX-interacting protein-like (*MLXIPL*, OMIM 605678) being the main candidate.[9] Other genes coding for the general transcription factor IIi (*GTF2I*, OMIM 601679) and the GTF2I repeat domain containing 1 (*GTF2IRD1*, OMIM 604318) could not be ruled out.[10] The metabolic phenotype of single-gene knock-out (KO) mouse models also contributed to associate glucose alterations to the *Mlxipl* [11] and syntaxin 1a (*Stx1a*, OMIM 186590) genes.[12] Moreover, *Mlxipl* KO mice presented reduced lipogenesis and a reduction in adipose tissue.[11]

We have further studied the metabolic alterations in a cohort of patients with WBS as well as in several mouse models to establish potential gene candidates. We have identified novel metabolic alterations, including relative hypotriglyceridemia and hyperbilirubinemia in a high proportion of individuals with WBS, and searched for genetic variants that could act as modifiers for the expression of these metabolic phenotypes. The diagnosis and management of these metabolic alterations could help prevent potential long-term complications in these patients.

MATERIALS AND METHODS

Subjects and metabolic data

Biochemical and hormonal parameters were analysed in a cohort of 154 individuals with WBS with well-characterised 7q11.23 deletions (see online supplementary table S1). Only patients with common recurrent deletions were included in our study; 89% (137/154) had a 1.55 Mb deletion, while 11% (17/154) carried a 1.83 Mb deletion.[4 13]

The majority of these patients were visited in two large clinical centres in Spain (88/154), and data were also collected from other multiple hospitals in Spain (33/154), Brazil (17/154), Portugal (14/154) and Argentina (2/154). Patients included in the study had a mean age of 12.98±9.03 years (range from 0.2 to 47 years), 80 men (12.54±9.11 years) and 74 women (13.45 ±8.98 years). Individuals younger than 18 years old were considered children (n=116), while individuals 18 years of age and older were classified as adults (n=38).

All parameters were measured at the patient's referral medical centre, following the procedures established by each laboratory. We used common reference intervals for each parameter to calculate z-score values and determine patients with outlier values. We also analysed our data by grouping them in centiles. The reference intervals for most laboratory tests with a normal distribution correspond to the 2.5 and 97.5 centiles (mean±1.96 SD), in which the central 95% of results are obtained from healthy individuals.[14] For most biochemical parameters, we used published reference values from the Spanish population.[15 16] For bilirubin levels, we used the reference values of the medical centres from which we had more patients. To detect possible biases related to the different diagnostic laboratories, we compared the z-score values and the indexes obtained by the medical centre of origin.

Polymorphism genotyping

Genetic variants previously reported to be associated with triglyceride or total bilirubin (TB) concentration were selected. The dinucleotide repeat $(TA)_{6-7}$ polymorphism in the TATA Box of the UDP-glycosyltransferase 1 family, polypeptide A1 (*UGT1A1,* OMIM 191740) gene promoter (rs8175347) was studied in 79 patients with WBS with bilirubin levels available, as well as in population controls (n=94), as previously reported.[17] We also studied a non-synonymous SNP (rs4149056) in the solute carrier organic anion transporter family, member 1B1 gene (*SLCO1B1,* OMIM 604843) associated with serum bilirubin levels,[18] and an intronic SNP (rs799160) at *MLXIPL* associated with triglyceride levels.[19] SNPs were genotyped using the Sequenom MassArray iPLEX system (Sequenom). Two HapMap samples and a trio were included in the assay for quality control, and no discordant genotypes were found.

For association analyses, we compared the allelic frequencies of extreme phenotypes. In case of triglyceride levels, cases were individuals with WBS with values below the 5th centile and controls were individuals with WBS with values above the 50th centile. Regarding bilirubinemia, cases were individuals with WBS with values above the 97.5th centile and controls were individuals with WBS with values below the 25th centile.

Animal models

The study was performed in accordance with the ARRIVE guidelines for the reporting of in vivo experiments (http://www.nc3rs.org/ARRIVE). Four groups of mice were used, all bred on a majority C57BL/6J background (97%) (see online supplementary figure S1): complete deletion (CD) mice bearing a heterozygous 1.1 Mb deletion of the orthologous WBS locus from *Gtf2i* to *Fkb6*,[20] mice with approximately half deletions of the interval, called the distal deletion (DD) (0.67 Mb from *Limk1* to *Trim50*) and the proximal deletion (PD) (0.45 Mb from *Gtf2i* to *Limk1*),[21] and the wild-type (WT) littermates as controls. Tail clipping was performed within 4 weeks of birth to obtain DNA using standard protocols and determine the genotype by a Multiple Ligation-dependent Probe Amplification assay (primers[22] in online supplementary table S2).

Metabolic analyses in mice, histological and anthropometric analyses are described in detail in online supplementary material and methods.

Candidate-gene expression studies

Liver samples were obtained from four mice per genotype at 26 ±1.1 weeks old. RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 1 µg total RNA using random hexamers and SuperScript II RNase reverse transcriptase (Invitrogen). The expression of *Ugt1a1* and *Rps28* (house-keeping control gene) were evaluated by qRT-PCR with primers spanning exons (see online supplementary table S2) and the Power SYBR Green PCR Master Mix using the 7900 HT Fast Real Time PCR System (Applied Biosystems). Samples were analysed in triplicates in three independent experiments and were discarded when the variation coefficient was >20%.

Statistical analyses

Statistical analysis was performed using the package SPSS V.19.0 according to the characteristics of each variable. Specifically χ^2, Student t test, analysis of variance with a post hoc least significant difference (LSD) comparison between multiple groups and non-parametric tests were used when needed. A p value <0.05 denoted the presence of statistically significant differences. Bonferroni's correction was performed for the metabolic analyses in mice.

RESULTS

Metabolic disturbances in individuals with WBS

Significant differences with respect to the expected values were observed for the following biochemical and hormonal parameters: TSH, glucose, triglyceride, cholesterol, TB, direct bilirubin (DB), indirect bilirubin, transferrin, and total protein and albumin levels (table 1 and see online supplementary table S1).

Subclinical hypothyroidism

Subclinical hypothyroidism (defined by mild TSH elevation with normal T3/T4 levels) was present in 31.3% of patients (26/83), with no gender differences. The values followed a bimodal distribution, with a second peak at the 97.5th centile (see online supplementary figure S2). There were 20 patients with values above 2.5 SDs. When compared by age, these patients were significantly younger (9.35±7.22 years) than the rest of the cohort with TSH values available (15.02±9.94 years) (p=0.021). There was a significant correlation between age and TSH values (r(81)=−0.356, p=0.001) and z-score value (r(81)=−0.356, p=0.001). Increased TSH levels occurred significantly more frequently in children than in adults with WBS (see online supplementary table S3). Three patients with congenital hypothyroidism and one with autoimmune thyroiditis were excluded from the analysis.

Table 1 Biochemical parameters studied in individuals with WBS

Biochemical parameter	Total individuals assessed no. (%)	Z-score (mean±SD)	Centiles (%)		
			<p2.5 (%)	p2.5–97.5 (%)	>p97.5 (%)
TSH	83/154 (53.9%)	1.158±1.78	0	68.7	31.3
Glucose	151/154 (98.1%)	0.642±2.261	2.0	90.7	7.3
Triglycerides*	115/154 (74.7%)	−0.146±0.887	18.3*	79.1	2.6*
Total cholesterol*	129/154 (83.8%)	−0.194±0.978	12.4*	83.7	3.9*
Total bilirubin	89/154 (57.8%)	0.499±2.47	1.1	78.7	20.2
Direct bilirubin	53/154 (34.4%)	−0.161±1.26	5.7	86.8	7.5
Indirect bilirubin	53/154 (34.4%)	2.08±5.09	0	66	34
Iron	70/154 (45.5%)	0.715±2.471	7.1	66.7	21.4
Transferrin	69/154 (44.8%)	0.378±0.851	0	95.7	4.3
Total protein	88/154 (57.1%)	1.13±1.25	0	72.7	27.3
Albumin	73/154 (47.4%)	0.96±0.886	0	89	11

Mean Z-score value, percentage of individuals below the 2.5th centile, between the 2.5th and 97.5th centile and above the 97.5th centile.
*For triglycerides and total cholesterol, percentage of individuals below the 5th centile, between the 5th and 95th centile and above the 95th centile.
TSH, thyroid-stimulating hormone; WBS, Williams–Beuren syndrome.

Hyperglycaemia

Basal glucose plasma levels were increased above the 97.5th centile in 7.3% of patients (11/151), 9 children (8%) and 2 adults (5.3%) (table 1). Only one of these patients had a diagnosis of diabetes mellitus type 1. The histogram of glucose centiles followed a normal distribution, with a small peak at 95–97.5th centiles (see online supplementary figure S3).

Hypotriglyceridemia and lipid profile

Triglyceride plasma levels were decreased in individuals with WBS; the values were arranged in a bimodal distribution with a first peak at the 2.5–5th centile and a second at the 25–50th centile (figure 1A). Specifically, 18.3% (21/115) of patients with WBS had levels below the 5th centile. There was no relationship between triglyceride levels and body mass index. Only 2.6% of patients (3/115) had hypertriglyceridemia and two of them presented associated hypothyroidism. The mean z-score value for triglyceride levels in our cohort of patients with WBS was below the reference interval mean (−0.146±0.887) (table 1). The distribution of the z-score values followed a normal distribution with a shift towards the left (figure 1B).

Cholesterol levels followed a bimodal distribution when analysed by centiles or z-score values (see online supplementary figure S4). Only one patient had total cholesterol values above the 97.5th centile. Cholesterol levels were below the 5th centile in 12.4% (16/129) of patients.

Hyperbilirubinemia

TB levels were increased in 20.2% (18/89) of patients with WBS. Two previously described patients with portal hypertension were excluded from the analysis,[23] as well as a patient diagnosed with beta-thalassaemia minor. TB levels followed a bimodal distribution, with a second peak at the 97.5th centile and a median between the 25th and 50th centiles (figure 2). There were 10 individuals with values above 3 SD (z-score range 3.5–15.78), with equal gender distribution. Indirect hyperbilirubinemia was present in 34% (18/53) of individuals with WBS and the distribution was also bimodal, while DB levels were only increased in

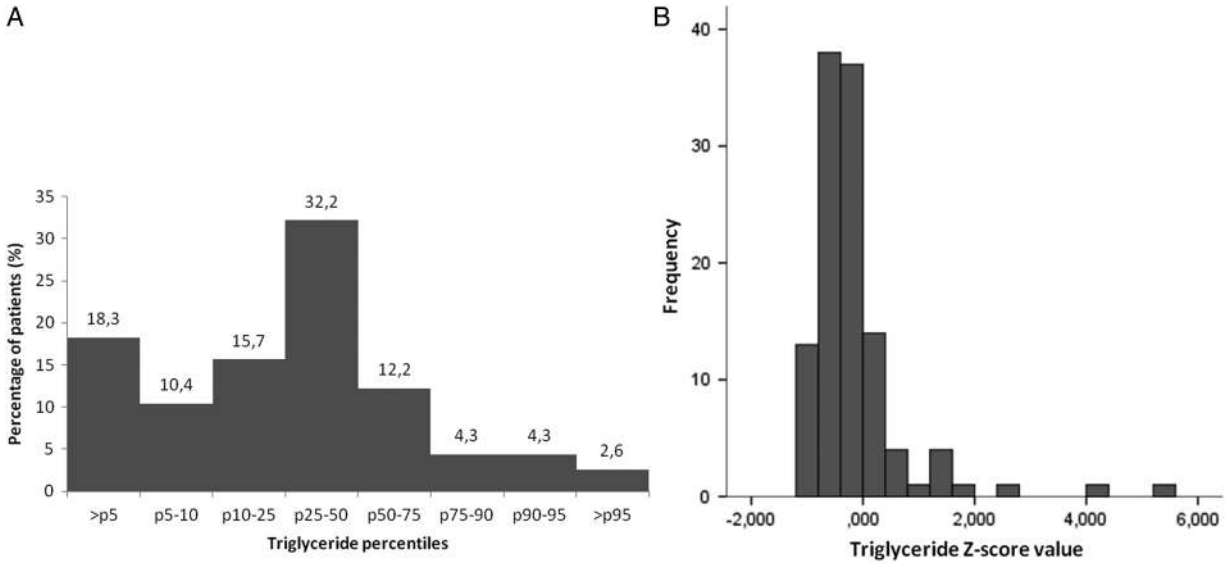

Figure 1 (A) Histogram of triglyceride plasma levels in centiles in individuals with Williams–Beuren syndrome. (B) Histogram of the distribution of triglyceride z-score values.

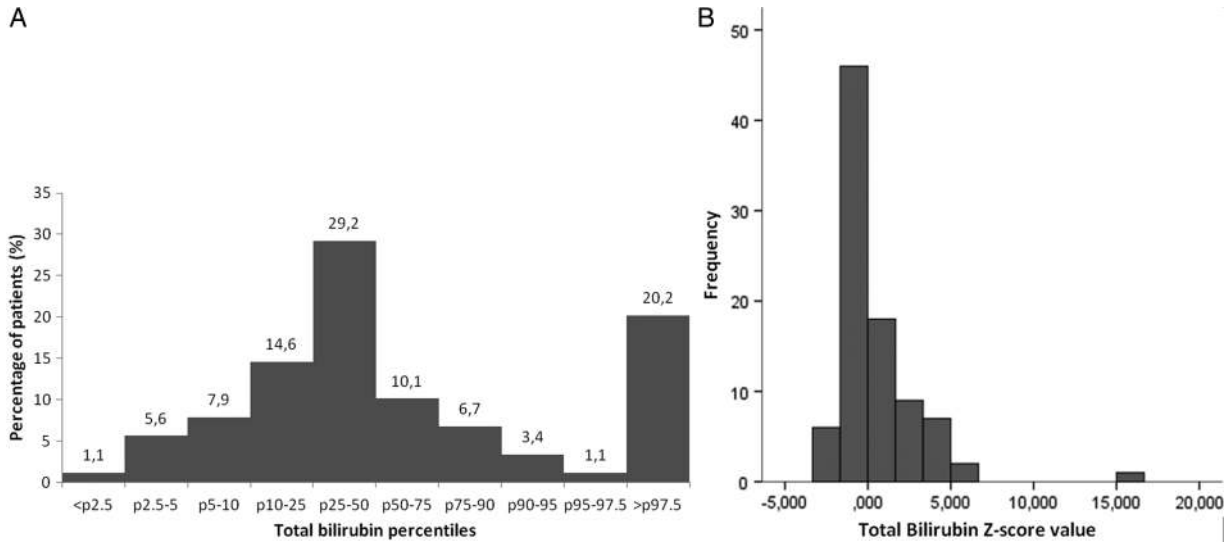

Figure 2 (A) Histogram of total bilirubin plasma levels in centiles in individuals with Williams–Beuren syndrome. (B) Histogram of total bilirubin z-score values.

7.5% (4/53) of WBS cases. There were also five individuals with normal TB levels and an increase of either indirect bilirubin (IB) or DB alone. Hepatic function parameters (aspartate aminotransferase (AST), alanine transaminase (ALT), gamma glutamyl transpeptidase (gGT)) were normal in all cases with hyperbilirubinemia but one, who had a slight increase in AST and ALT but normal gGT levels. Out of the 14 individuals with hyperbilirubinemia and fractionated bilirubin levels available, unconjugated hyperbilirubinemia (DB/TB ratio <30%) was present in 28.6% (4/14) while mixed hyperbilirubinemia (DB/TB ratio 30–70%) was found in the remaining 71.4% (10/14). None presented a conjugated hyperbilirubinemia (DB/TB ratio >70%). Individuals with hyperbilirubinemia were significantly older than those with normal bilirubin levels (20.21±10.31 years vs 11.82 ±8.40 years, respectively. p=0.001). TB and IB levels were more frequently increased in adults compared with children (see online supplementary table S3).

Abnormal protein and iron levels

A high proportion of individuals with WBS displayed elevated total protein (27.3%) and elevated albumin levels (11%). Only 7 of the 22 cases with hyperproteinemia and albumin levels available had an elevation of this parameter. Total protein levels followed a bimodal distribution with a second peak at 97.5th centile.

We also found alterations in iron metabolism, with 21.4% (15/70) of individuals showing iron concentrations above the 97.5th centile. Haemoglobin and haematocrit levels were normal in all cases. Iron concentration had a bimodal distribution with a first peak at the 25–50th centile and a second at >p97.5th centile. Iron z-score values followed a normal distribution with a shift towards the right.

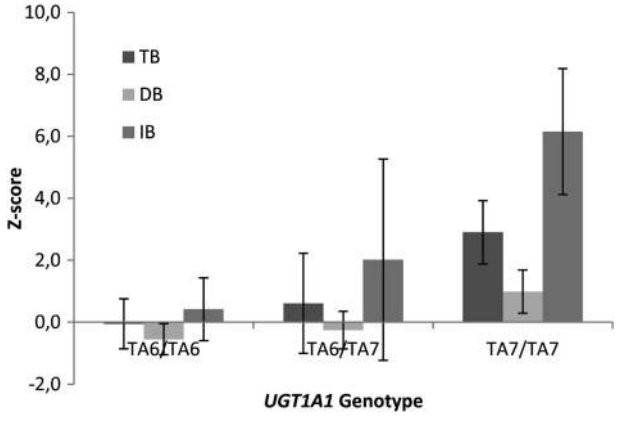

Figure 3 Total bilirubin (TB), direct bilirubin (DB) and IB z-score values with respect to *UGT1A1* genotype (mean±SD). Significant differences were found between genotypes with respect to TB (F(2,76)=6.412, p=0.003), DB (F(2,49)=5.497, p=0.007) and IB (F(2,49)=4.397, p=0.018).

Correlations between metabolic disturbances

TSH z-score values correlated significantly with TB z-score values in an inverse manner (r(66)=−0.328, p=0.006). Triglyceride z-score value correlated significantly with TB z-score (r(78)=−0.220, p=0.050) and with total protein z-score value (r(79)=−0.321, p=0.003) in an inverse manner. Cholesterol z-score values also correlated with DB z-score values (r(51)=−0.436, p=0.001). As expected, total protein z-score values correlated significantly with albumin z-score values (r(71)=0.666, p<0.001). We did not find any correlation of the biochemical alterations detected and molecular data at 7q11.23, either the size or the parental origin of the deletion.

Genetic modifiers of the lipid and bilirubin disturbances

In order to test whether genetic variants at the non-deleted allele might be responsible for the variation in the lipid profile of individuals with WBS, we genotyped an SNP of the *MLXIPL* gene previously associated with hypotriglyceridemia.[19] We compared allelic frequencies of individuals with WBS with triglyceride values below the 5th centile (n=21) with those with triglyceride values above the 50th centile that were considered controls (n=24). No significant differences were found between cases and controls (see online supplementary table S4).

We also tested whether the hyperbilirubinemia observed in patients with WBS was related to known genetic susceptibility factors: a dinucleotide repeat polymorphism at the *UGT1A1* promoter and an SNP at the *SLCO1B1* gene.[18 24] The *UGT1A1* polymorphism was studied in 79 patients with WBS with bilirubin values available and 94 Spanish population controls (no

Table 2 Metabolic parameters analysed in WBS mice models at 25 weeks of age

	WT	CD	DD	PD
Age (weeks)	26.27±0.8	26.11±0.6	25.7±1.25	25.44±1.74
Triglycerides (mg/dL)	99.4±9.97	104.1±21.67	83.91±12.41	84.38±14.4
(no. mice)	(8)	(8)	(7)	(4)
Total cholesterol (mg/dL)	117.1±28.16	123.6±6.61	131.48±16.8	124.6±18.6
(no. mice)	(15)	(7)	(10)	(10)
Total bilirubin (mg/dL)	0.617±0.343	0.506±0.211	0.45±0.25	0.471±0.221
(no. mice)	(13)	(4)	(6)	(6)
Direct bilirubin (mg/dL)	0.419±0.36	0.303±0.15	0.158±0.086	0.202±0.126
(no. mice)	(13)	(4)	(6)	(6)
Indirect bilirubin (mg/dL)	0.198±0.137	0.204±0.117	0.293±0.200	0.269±0.116
(no. mice)	(13)	(4)	(6)	(6)
Total protein (mg/dL)	4791±314	4747±422	4894±333	5015±161
(no. mice)	(14)	(12)	(10)	(12)
Glucose (mg/dL)	173.9±49.9	167.8±41.1	189.6±59.9	157±52.8
(no. mice)	(12)	(8)	(8)	(3)
TSH (uIU/mL)	0.142±0.014	0.116±0.063	0.193±0.032	0.223±0.054*
(no. mice)	(5)	(3)	(3)	(6)

All values are mean±SD. Asterisks represent significant differences of at least p<0.006 (after Bonferroni's correction) compared with WT mice.
CD, complete deletion; DD, distal deletion; PD, proximal deletion; TSH, thyroid-stimulating hormone; WBS, Williams–Beuren syndrome; WT, wild-type.

bilirubin levels available). Allelic frequencies were not significantly different in individuals with WBS and controls (see online supplementary table S5). *UGT1A1* genotype was significantly associated with elevated plasma concentrations (>2.5SD) of TB (p<0.001) and IB (p<0.001) in WBS (DB (p=0.055)). The penetrance of hyperbilirubinemia in individuals with WBS homozygous for the *UGT1A1*-promoter allele [$(TA)_7$] was of 72.7%. Hyperbilirubinemia was present in 13.3% of [$(TA)_6$/$(TA)_7$] heterozygous individuals with WBS, either unconjugated or mixed hyperbilirubinemia, and 13.2% of [$(TA)_6$] homozygous individuals with WBS, all with unconjugated hyperbilirubinemia (figure 3). Although there was a significant correlation between *UGT1A1* genotypes and bilirubin levels, specifically increased bilirubin in patients homozygous for [$(TA)_7$], there was a great variability of bilirubin levels among individuals with the same *UGT1A1* genotype. However, no significant differences in the allelic frequencies of the *SLCO1B1* SNP genotypes were observed in WBS with respect to bilirubin levels.

Metabolic disturbances in WBS mouse models

TSH was measured in all mouse models at 25 weeks of age (table 2). Only PD mice had a significant increase in TSH levels compared with WT (p=0.004). Basal blood glucose level at 25 weeks and the intraperitoneal glucose tolerance test in 11-week-old mice showed no significant differences between WT animals and deletion mouse models (figure 4A). A morphological analysis of the pancreatic Langerhans islets revealed that CD mice had a higher percentage of smaller islets compared with the WT (66% in CD vs 48.8% in WT) and less bigger islets (0.51% in CD vs 6.51% in WT) (figure 4B).

Triglyceride plasma concentration was significantly decreased only in the DD and PD mice at 4.5 weeks of age compared with WT (p=0.003 and 0.005, respectively), a decrement that was milder and non-significant at 26 weeks of age (table 2). Total cholesterol, high-density lipoprotein and low-density lipoprotein levels were not significantly different in any group of animals at any age (table 2 and see online supplementary table S6).

An anthropometric analysis was done at 25 weeks in mice; whole body, liver, perirenal and gonadal fat weight were recorded (see online supplementary table S7). PD mice had significant increases in total body weight (p=0.018), liver weight with respect to total body weight (p=0.002) and total fat (p=0.015) compared with WT.

Total protein levels were measured in mice at 25 weeks (table 2). PD mice had increased concentration compared with WT, although it did not reach statistical significance after Bonferroni's correction.

Bilirubin levels were also studied at 4.5 and 25 weeks in mice with no significant differences in absolute values between groups of animals. WT mice had almost the same TB values at both time points, although a relative increase of DB and decrease of IB was observed at 25 weeks, resulting in increased DB to TB ratio (DB/TB) (41.9%±12.1% vs 61.2%±22.3%). In the case of DD mice, TB decreased at 25 weeks maintaining a similar DB/TB ratio at both time points (31.7%±10.8% vs 38.4%±16.6%). For PD mice, all values increased at 25 weeks except the DB/TB ratio that was similar at both time points (44.2%±14.3% vs 41.9%±12.1%).

In order to test for a putative differential compensatory regulation of the *Ugt1a1* gene in the different mice, we measured its relative expression in RNA isolated from mice liver by qRT-PCR. No differences were found with respect to *Ugt1a1* expression levels between the four mouse genotypes studied (see online supplementary figure S5).

DISCUSSION

We describe several unreported biochemical alterations relatively frequent in children and adults with WBS, such as hypotriglyceridemia, increased bilirubin levels and increased total protein and albumin levels, and we document the previously described subclinical hypothyroidism and glucose intolerance.

Subclinical hypothyroidism has been previously reported in WBS with a frequency ranging from 15% to 31.5%.[7 25 26] In our cohort, 31.3% of individuals with WBS presented subclinical hypothyroidism. Patients presenting this alteration were significantly younger than patients with normal TSH plasma concentrations. A suggested mechanism for its high prevalence in WBS is the immaturity of the hypothalamic–pituitary–thyroid axis that may associate some degree of thyroid hypoplasia and spontaneously resolves at increasing age.[26] Antithyroid autoimmunity is rarely seen, just a single case in our cohort. Thus, hormonal replacement therapy is not usually required and

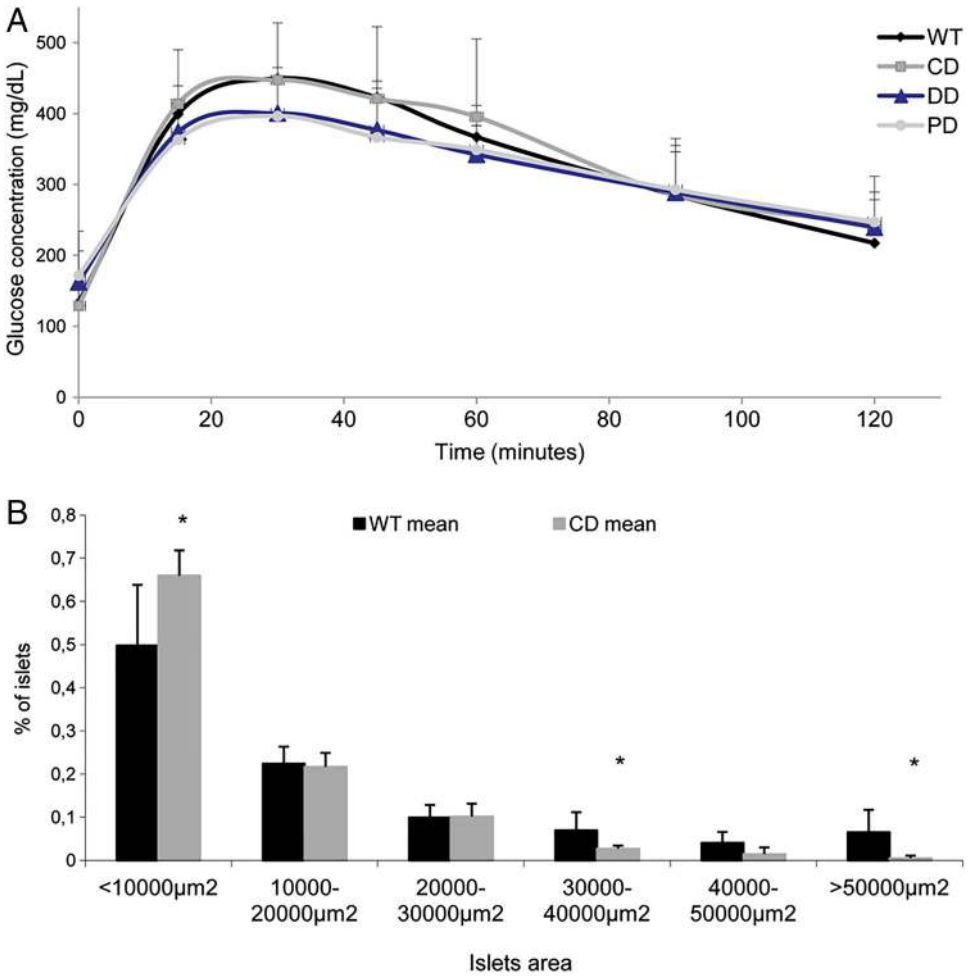

Figure 4 (A) Intraperitoneal glucose tolerance test. Results represent mean±SD (n=8–16). (B) Langerhans islets area analysis. Results represent mean±SD (n=3–6).

should be reserved for patients with worsening thyroid function.[25] The finding of elevated TSH values only in mice with deletion of the PD interval suggests that haploinsufficiency for one or more genes located in this interval could contribute to this developmental phenotype of the thyroid axis.

Impaired glucose tolerance has been reported in 75% of adult individuals with WBS.[8] We found slight basal hyperglycaemia in 7.3% of individuals with WBS and a single case with diabetes mellitus type I. Considering the young mean age of these patients with basal hyperglycaemia in our cohort (10.2±8.9 years), the findings are quite relevant and further support the recommendation for an oral glucose tolerance test in individuals with WBS in the second or third decade of life in order to detect and treat this disturbance.[8] Neither hyperglycaemia nor glucose intolerance was seen in the three WBS mouse models studied at 11 weeks of age. However, morphological alterations in pancreatic Langerhans islets were observed in CD mice, with a significant reduction in the islets size. A similar morphological alteration, with smaller islet area and losses of beta and alpha cells, has been reported in patients with type 2 diabetes.[27] The reduction of islet size may not have a clinical 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.

Triglyceride levels were below the 5th centile in 18% of individuals with WBS in our cohort, with a global distribution shifted towards the left. However, none of them presented extremely low triglyceride values below 2.5 SD. Total cholesterol was also distributed in a bimodal curve with only one patient presenting values above the 97.5th centile, in contrast to the high prevalence of hypercholesterolemia in the adult and paediatric Spanish population.[28 29] Therefore, the most common lipid profile in WBS is hypotriglyceridemia with low-normocholesterolemia, which is associated with low risk for cardiovascular disease. Nevertheless, given the high prevalence of other cardiovascular risk factors, including cardiac and vascular stenoses, hypertension and glucose intolerance, all individuals with WBS should have periodic evaluations of the lipid profile to intervene if necessary.

An excellent candidate gene for the lipid profile in WBS is *MLXIPL*, invariably deleted in patients. *MLXIPL* encodes a basic-helix-loop-helix leucine zipper transcription factor of the Myc/Max/Mad superfamily[30 31] that preferentially regulates triglyceride synthesis and storage.[32 33] Several SNPs at or near *MLXIPL* have been associated with high and low triglyceride plasma concentration in different populations.[19 34–36] However, we ruled out any association of the variable expression of hypotriglyceridemia in patients with WBS with a common SNP in the non-deleted copy of *MLXIPL*. Homozygous mice KO for *Mlxipl* present decreased lipogenesis, low plasma free fatty acids and reduced adipose tissue mass, although heterozygous mice were not studied.[11] We observed significantly decreased triglyceride levels at 4.5 weeks of age in two of the mouse models analysed harbouring non-overlapping deletions, DD and PD. Unfortunately, CD mice were not studied at this time point due

to the unavailability of enough sample.[37] At 26 weeks of age, hypotriglyceridemia only persisted in DD mice, hemizygous for *Mlxipl*. Therefore, the major cause for hypotriglyceridemia in WBS and mouse models is, most likely, haploinsufficiency at *MLXIPL*, although the more penetrant phenotype at early ages could be modified by other genes of the interval, as well as by environmental factors such as diet.[11]

The somehow inconsistent findings of some biochemical phenotypes in partial deletion mice absent in the CD mice are relatively common in mouse models of segmental or complete aneuploidy. When phenotypes of microdeletions are the result of interactive effects of the haploinsufficient genes, there may be compensatory effects with partial deletions absent in full deletions.[20 38 39] In addition, the failure to show relevant phenotypes in mouse models with similar genotypes to humans underscores the physiological differences between mice and humans. Therefore, a simple explanation of how haploinsufficiency of some deleted genes causes the observed phenotypes is neither adequate nor possible with the current data.

Mildly elevated TB levels were observed in a relatively high proportion of individuals with WBS (20.2%). Hyperbilirubinemia may indicate increased degradation of haemoglobin (haemolytic disease), reduced transport of bilirubin into the liver, reduced glucuronidation of bilirubin or hepatobiliary disease. In the absence of haemolysis or underlying liver disease, the mild mostly unconjugated hyperbilirubinemia of individuals with WBS is consistent with an additional diagnosis of Gilbert syndrome (OMIM 143500). Gilbert syndrome, characterised by intermittent mild jaundice, has a prevalence in the range of 6–8%.[24 40–43] It is mainly caused by a hypomorphic allele at the promoter of the *UGT1A1* gene, encoding a glucuronosyltransferase enzyme responsible for the glucuronidation essential for bilirubin excretion.[18 24 44] The penetrance is incomplete and varies depending on the criteria used to define the phenotype, being around 40% for the homozygous hypomorphic allele. Genotype frequencies at *UGT1A1* in our WBS cohort were similar to those reported in the Spanish population,[45] and bilirubin levels correlated with *UGT1A1* genotype, as previously reported.[24 46 47] No association was found with *SLCO1B1*,[18] coding for an hepatic transporter in the basolateral membrane of hepatocytes for bilirubin.[18 48] Therefore, the increased frequency of unconjugated hyperbilirubinemia or Gilbert syndrome in WBS, threefold that of the general population, is partly due to increased penetrance of homozygous *UGT1A1* hypomorphic alleles (72.7%). However, 13.2% of patients with WBS homozygous for the functional allele also had hyperbilirubinemia, and normal bilirubin levels and normal expression of *Ugt1a1* in hepatic tissue were found in all mouse models tested, implying that *UGT1A1* is not the only cause of hyperbilirubinemia in patients with WBS. We found that TB z-score values were inversely related with triglyceride z-score values, suggesting common pathogenic mechanisms for the lipid and bilirubin alterations, as described by other studies.[49 50]

Additional abnormalities were found in our cohort, including elevated total protein in 27.3%, elevated albumin levels in 11%, relatively increased transferrin and elevated iron in 21.4% of patients with WBS. As for the other metabolic parameters, a limitation of our study is the relatively small sample size, common when studying endophenotypes in rare diseases. Further studies are needed to confirm these data and better explain the mechanisms underlying the high frequency of these metabolic alterations.

Our data indicate that several unreported biochemical alterations are relatively common in WBS and should be studied in greater detail in order to better define clinical guidelines and prevent potential long-term complications, such as the known consequences of clinical hypothyroidism or diabetes. The most common metabolic profile observed in WBS, with hypotryglyceridemia and low-normal cholesterol, is indeed associated with an a priori low risk for cardiovascular disease. Given the high cardiovascular risk of these patients due to the developmental and functional anomalies of the elastin deficiency, this metabolic profile could somehow provide a compensatory mechanism. Awareness about this metabolic profile of WBS is relevant to avoid unnecessary studies and interventions, as well as to better identify relevant changes that could have a negative impact on the potential cardiovascular complications.

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Contributors MGP-V, VC and LAP-J: conceived and designed the experiments. MDC and LAP-J: phenotype patients. MGP-V, RF, CB, MS-P and VC: performed the laboratory experiments. MGP-V and LAP-J: analysed the data. MGP-V, VC and LAP-J: wrote the paper.

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Competing interests LAP-J is scientific advisor of qGenomics, a privately held company that provides genomics services to the scientific and medical community.

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