WILLIAMS SYNDROME PREDISPOSES TO VASCULAR STIFFNESS MODIFIED BY ANTI-HYPERTENSIVE USE AND COPY NUMBER CHANGES IN *NCF1*

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Supplemental Methods:

Study examination and historical data acquisition
Each individual was examined by a clinical geneticist experienced in the diagnosis of WS (BP and BK) and was felt to possess physical features consistent with WS. In addition to examination of facial features, individuals underwent cardiovascular examination including cardiac auscultation, assessment of peripheral pulses by palpation and auscultation for abdominal bruits. Parents/caregivers answered questions about cardiovascular history including presence of stenosis, hypertension, and diabetes. A subgroup was also asked about stroke history. Release of records was obtained for the most recent echocardiogram. Historical information obtained by questionnaire was validated in medical records when possible.

DNA collection and Confirmation of WS Diagnosis:
Molecular confirmation of the WS diagnosis was sought through review of clinical testing results (ELN FISH or chromosomal microarray). If clinical testing was not available, elastin deletion was confirmed by qPCR (research).

Saliva was collected in Oragene saliva kits (DNA Genotek, Kanata, Ontario, Canada) at the time of in-person assessment. DNA was prepared from the saliva sample according to manufacturer's instructions. We performed ELN copy number analysis on a Viia7™ Real-Time PCR System (Applied Biosystems, (ABI), Foster City, CA) using a TaqMan Copy Number Assay. Probes (Elastin (ABI, #4400291, labeled FAM) and RNase P, (the reference gene, ABI, #4403326, labeled VIC)) were used in the reaction with 25ng of genomic DNA and TaqMan Genotyping Master Mix. PCR parameters were as specified in the manufacturer's Copy Caller protocol (ABI). DNAs from four unaffected individuals (CN= 2) and one WS individual with a known positive *ELN* FISH (CN=1) were used as controls. A no DNA control failed to amplify. Copy Caller software (Applied Biosystems) was used to analyze the data and assign copy number. All consented individuals have phenotypically and molecularly confirmed WS (See Figure S1 for representative data.)

NCF1 gene and pseudogene copy number determination
Saliva was collected and DNA purified as above. To calculate the *NCF1* gene copy number, both the genes and pseudogenes (which differ by two bp, pseudogenes have ΔGT at the beginning of exon 2) were amplified together using PCR primers surrounding the divergent region. Amplification primers used were NCF1F 2LB2 (5'--GTGCACACAGCAAAGCCTCT--3') and NCF1R 2RB2 (5'--CTAAGGTCCTTCCCAAAGGGT—3'). Following PCR amplification, the product was gel purified and Sanger sequenced. At the ΔGT, the gene and pseudogene sequences diverge and the peak heights (gene and pseudogene) are determined from the tracings for the next 27 bases using Applied Biosystems Sequence Scanner 1.0 software. Pseudogene:Gene (P:G) peak height ratios are calculated for each base and averaged. From the raw *NCF1* P:G ratio, pseudogene and gene copy number were assigned using the ratio table in Table S1.

Using the relative peak height method, the most commonly observed pattern in those without WS is 4 pseduogenes:2 genes, while individuals with typical WS deletions

(1.5 or 1.8 MB) display the absence of one or two pseudogenes and/or genes, respectively, perturbing the 4:2 ratio. Of note, 7 of 103 WS participants were found to have more than 2 copies of *NCF1*, but fewer copies of the pseudogenes, a finding reported previously in WS[1] and control populations[2] and thought to be due to inherited gene conversion between the gene and pseudogenes. Copy number frequencies are reported for individuals as either one (CN=1) or two or more (CN≥2) functional *NCF1* copies.

Vascular Phenotype assessment (Hypertension and PWV)
Hypertension: History of hypertension was ascertained in 101/103 participants. Survey responses indicated inadequate information existed to determine hypertension status in 10/103 WS participants. Eight of these did not report use of anti-hypertensive in their medication list and their blood pressure was normal in both arms on the day of in-person evaluation (<95%ile for age, gender and height in pediatric patients or<140/90 in adults); these individuals were treated as not having hypertension for the duration of the analysis. The last two remaining individuals had insufficient information to determine their blood pressure status and were excluded from analyses that required this parameter.

PWV: Applanation tonometry was used to calculate PWV in each participant. To do so, waveforms were recorded from the carotid and femoral arteries while EKG was recorded. PWV was calculated from these waveforms and measurements from standard landmarks ((sternal notch to arterial femoral site distance)-(sternal notch to carotid site distance)). PWV was determined using a SphygmoCor device (AtCor Medical, Sydney) for the WS-SAVE participants and the WUSM adult control groups and with the Pulse Pen (DiaTecne, Milan) in the Semmelweis pediatric control group. Published studies show high agreement for PWV between the SphygmoCor and the Pulse Pen[3].

References:

1. Del Campo M, Antonell A, Magano LF, Munoz FJ, Flores R, Bayes M, Perez Jurado LA. Hemizygosity at the ncf1 gene in patients with williams-beuren syndrome decreases their risk of hypertension. *Am J Hum Genet*. 2006;78:533-542.
2. Heyworth PG, Noack D, Cross AR. Identification of a novel ncf-1 (p47-phox) pseudogene not containing the signature gt deletion: Significance for a47 degrees chronic granulomatous disease carrier detection. *Blood*. 2002;100:1845-1851.
3. Kis E, Cseprekal O, Kerti A, Salvi P, Benetos A, Tisler A, Szabo A, Tulassay T, Reusz GS. Measurement of pulse wave velocity in children and young adults: A comparative study using three different devices. *Hypertens Res*. 2011;34:1197-1202.
4. Pober BR. Williams-beuren syndrome. *N Engl J Med*. 2010;362:239-252.
5. Reusz GS, Cseprekal O, Temmar M, Kis E, Cherif AB, Thaleb A, Fekete A, Szabo AJ, Benetos A, Salvi P. Reference values of pulse wave velocity in healthy children and teenagers. *Hypertension*. 2010;56:217-224.
6. McEniery CM, Yasmin, Hall IR, Qasem A, Wilkinson IB, Cockcroft JR. Normal vascular aging: Differential effects on wave reflection and aortic pulse wave velocity: The anglo-cardiff collaborative trial (acct). *J Am Coll Cardiol*. 2005;46:1753-1760.

Supplemental Tables and Figures:

Table S1: NCF1 genotyping. *NCF1* gene number was determined after assignment to a genotype bin based on the ratio of *NCF1* pseudogenes:gene peaks (P height/G height for each base, averaged over 27 bases). Nine non-WS controls were used. All controls have the expected 6 total NCF alleles. Of note, 7 of 103 WS participants and two of nine controls were noted to have more than 2 copies of *NCF1*, but fewer copies of the pseudogenes. This finding was reported previously in WS[1] and in control populations[2] and is thought to be due to inherited gene conversion between the gene and pseudogenes. Establishing a breakpoint between the 3:1 and 4:1 P:G bins requires knowledge of the deletion size (1.5 MB (5 total alleles) *vs* 1.8 MB (4 total alleles)). This analysis was not performed because both groups have an *NCF1* gene copy number of 1 and differentiation between the two would not affect further statistical analysis. DNAs from two individuals were not of sufficient quality to provide copy number information.

NCF1 Genotype Pseudogene:Gene	Ratio range	Number of Participants	
		WS	Control
2:3	0.68-0.78	5	0
3:3 or 2:2	1.04-1.07	2	2
3:2	1.35-1.95	47	0
4:2/2:1	1.96-2.15	0	7
4:1/3:1	2.4-4.19	47	0

Table S2: Characteristics of WS participants on which PWV was not achievable. PWV was attempted on 103 participants and 77 had successful attempts. The successful and unsuccessful groups of participants were compared for differences in age and body mass index (BMI) (t test) and percent history of hypertension, use of anti-hypertension medication, stenosis (any type), supravalvar aortic stenosis (SVAS), surgical SVAS and diabetes (Fisher's exact test). The unsuccessful group had a higher proportion of females ($p=0.02$) and had a higher BMI ($p=0.002$) than those in which PWV was achieved. In addition, the unsuccessful cohort had a higher rate of surgery for stenosis ($p=0.02$). With each of the remaining features evaluated, the unsuccessful cohort trended toward having more severe vascular disease but none of these comparisons reached statistical significance. These results suggest that the unsuccessful cohort is unlikely to have milder features that would unfairly bias results coming from the successful group. *denotes statistically significant results.

Table S2:

	Failed PWV Participants N=26 Average (range)	Successful PWV Paricipants N=77 Average (range)	p value
Age (years)	24.1 (7-54)	24.6 (7-62)	0.85
BMI	28.2 (10-48)	22.9 (13-40)	0.002*

	% (feature present/ total)	% (feature present/ total)	
Male (%)	27% (7/26)	53% (41/77)	0.02*
Hx of Hypertension	52% (13/25)	40% (30/76)	0.35
Hypertension Medication (any indication)	40% (10/25)	29% (22/76)	0.32
Hypertension Medication for HTN Diagnosis	35% (9/25)	25% (19/76)	0.31
Any Stenosis (%)	83% (20/24)	69% (49/71)	0.20
SVAS (%)	67% (16/24)	54% (38/70)	0.34
Surgical SVAS (%)	33% (8/24)	11% (7/65)	0.02*
Diabetes (%)	21% (5/24)	10% (7/73)	0.16

Table S3. Phenotypes of matched adult WS and controls. Mean age and BMI, as well as percent of participants with hypertension (HTN), diabetes, or anti-hypertensive use are shown for both the WS cohort and the matched adult control group. P values for the t test (age and BMI) or Fisher's exact test (HTN, HTN meds, and diabetes) are shown.

	Age	BMI	Hx of HTN (%)	Use of HTN Meds (%)	Diabetes (%)
Control	33.9	27.4	44	29	13
WS	33.7	25.7	49	34	18
p value	0.91	0.24	0.82	0.81	0.76

Table S4: Demographic data for the 77 WS-SAVE participants with quality PWV tracings. Data shown include the frequency of various phenotypes in the total cohort or in individuals of decade binned groups (≤10 years, 11-20 years, etc). BMI=Body Mass Index, HTN=Hypertension, SVAS=Supravalvar Aortic Stenosis. * Anti-hypertensive medications used include (beta blockers (8), ACE inhibitors/Angiotensin II receptor blockers (14), calcium channel blockers (3), diuretics (2), and alpha adrenergic blockers(1)). Five individuals used more than one anti-hypertensive (in each case, an ACE inhibitor or ARB were used in addition to a second medication (4 individuals) or two additional medications (1 individual). Anti-hypertensive medications were prescribed for blood pressure control in all but three individuals (1 used only an alpha adrenergic receptor blocker to treat attention deficit disorder, one received a beta blocker for anxiolysis, and one received an ACE inhibitor for afterload reduction for mitral valve prolapse with regurgitation).

Table S4

	Total cohort	≤10	11-20	21-30	31-40	41-50	≥51
Age (mean, years)	25 (n=77)	9 (n=10)	16 (n=23)	25 (n=23)	35 (n=12)	46 (n=6)	56 (n=3)
male	41/77	7/10	10/23	9/23	7/12	5/6	3/3
BMI (mean, range)	23, 13-40	19, 13-26	20, 14-30	26, 15-38	25, (18-40)	25, (19-30)	23.7 (17-30)
History of HTN	30/76	4/10	6/22	6/23	9/12	4/6	2/3
HTN Meds*	22/76	3/10	5/22	3/23	5/12	4/6	2/3
Meds for HTN	19/76	3/10	3/22	3/23	5/12	4/6	1/3
Any Stenosis	49/71	8/10	15/20	15/21	7/11	3/6	1/3
SVAS	38/70	6/10	11/20	12/21	7/11	3/6	0/2
Surgical SVAS	7/65	1/10	2/19	2/18	2/11	0/5	0/2
Diabetes	7/73	0/10	0/22	1/21	3/11	2/6	1/3
Known Stroke	2/36	0/2	0/15	0/7	2/6	0/4	0/1

Figure S1: Confirmation of WS diagnosis by ELN copy number. We performed ELN copy number analysis to confirm the WS diagnosis. DNA from four unaffected individuals (black bars) and one WS individual with a known positive *ELN* FISH (WS-400, CN=1, gray bar with white dots) were used as controls. Representative WS participants are shown as gray bars. A no DNA control failed to amplify. All consented WS individuals were shown to have molecularly confirmed WS.

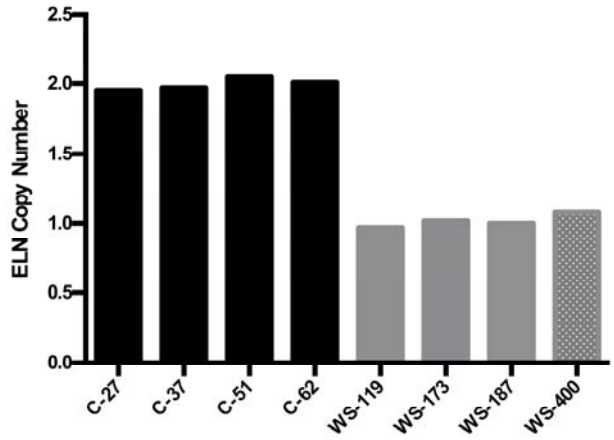

Figure S2: Depiction of the Williams deletion region. The WS critical region (blue) on chromosome 7 is flanked by three low copy number repeat regions, most often consisting of *NCF1* (purple) and one of its pseudogenes (NCF1B, green; NCF1C, red) [1, 4]. The majority of non-WS individuals in the general population have a total of 6 *NCF1* pseudogenes (P) + genes (G) in a P:G ratio of 4:2. The ratio is altered in WS, in both the 1.5 MB and less frequent 1.8 MB deletion. In individuals with the 1.5 MB deletion, either the *NCF1* gene (purple) or the *NCF1B* pseudogene (green) is deleted; this leads to a total of 5 P+G, with ratios of P:G 4:1 or 3:2. In individuals with the 1.8 MB deletion, the recombination occurs between the two pseudogene regions (green and red), leading to the deletion of the *NCF1* gene (purple) AND deletion of either the *NCF1B* (green) or the *NCF1C* (red) pseudogene; deletion of green and purple shown as example). This result in 4 total P + G with a P:G ratio of 3:1. Some individuals (WS and control) have more than 2 copies of *NCF1*, but fewer copies of the pseudogenes. This finding is thought to be due to inherited gene conversion between the gene and pseudogenes (not shown in the figure).

Figure S2

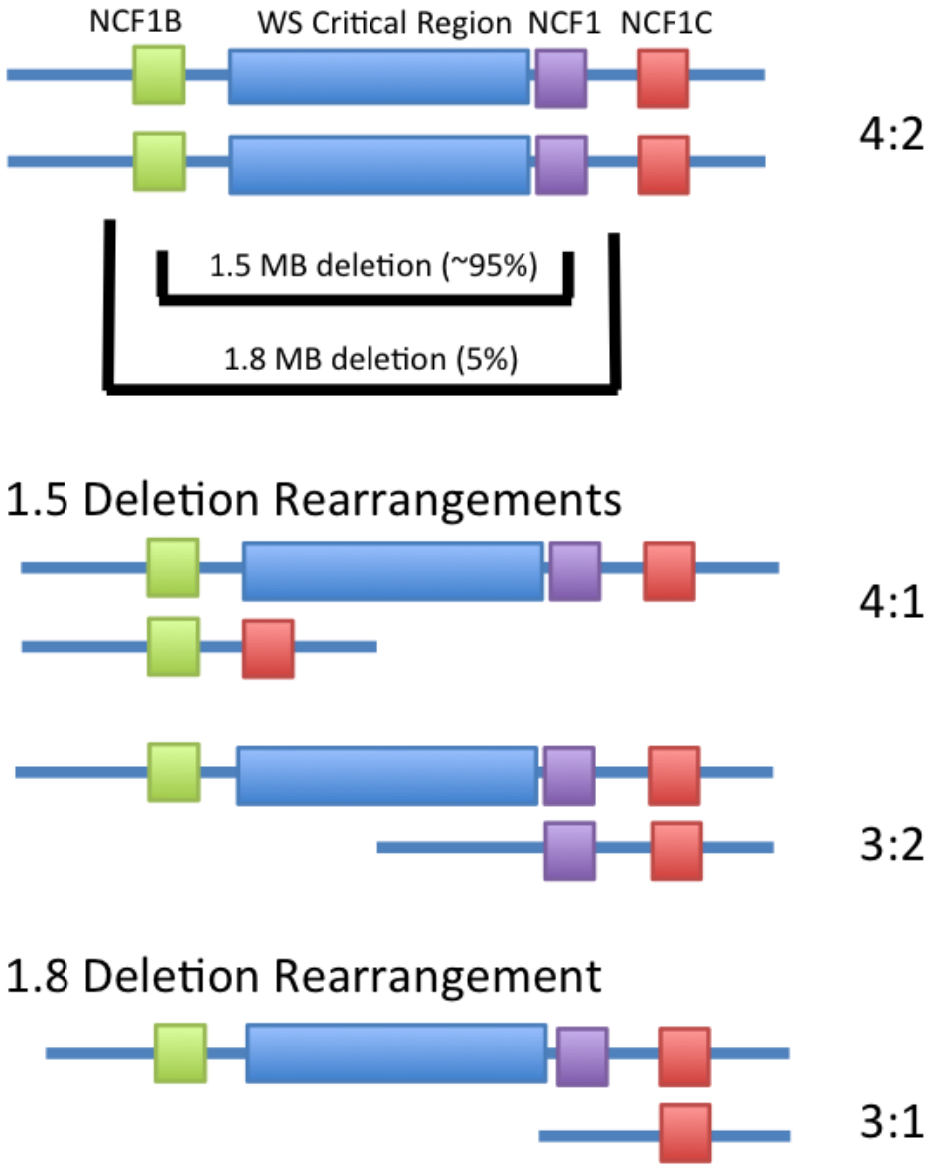
NCF1B
WS Critical Region
NCF1
NCF1C
4:2
1.5 MB deletion (~95%)
1.8 MB deletion (5%)
1.5 Deletion Rearrangements
4:1
3:2
1.8 Deletion Rearrangement
3:1

Figure S3: Individuals with WS have stiffer blood vessels than normal population controls. In panel A, raw PWV from the 77 participants with quality PWV studies are plotted against the age of the patient. Male WS participants are denoted as black squares. Female WS participants are grey circles. Normative data for pediatric patients[5] are noted by solid lines while adult normative data (derived from[6]) are shown by hatched lines. In both control groups, males are indicated by a blue line and females, by a red line. Most, but not all, individuals with WS have higher PWV than age/gender matched peers. To evaluate for possible differential effects of aging on PWV in WS relative to controls, regression was performed using the full data PWV set (n=77 adults and children, Panel B). This analysis showed higher PWVs in WS participants across the whole age distribution ($p < 0.0001$ for elevation) with no statistical difference in the rate of PWV increase with age compared to controls (p=NS).

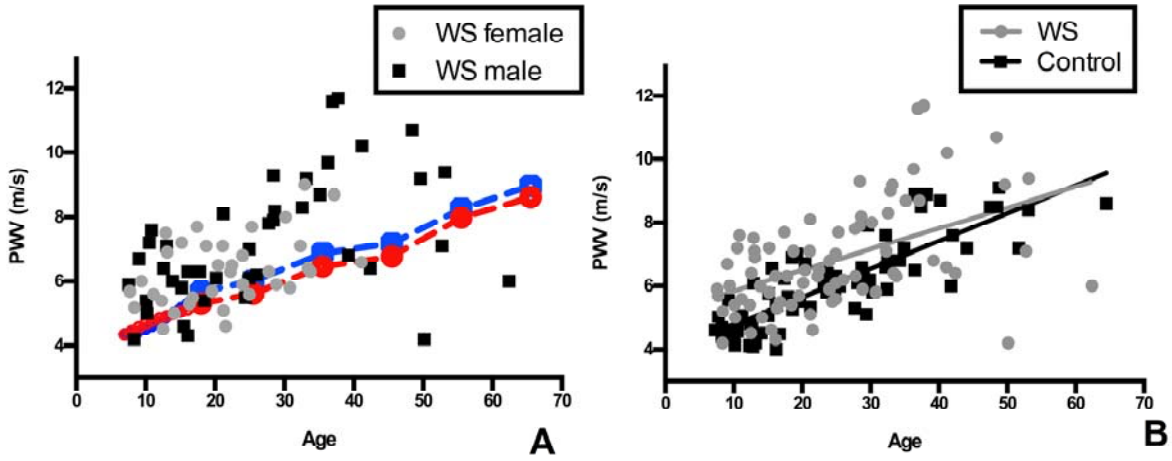

Figure S4: Lack of influence of diabetes on the severity of vascular stiffness in WS. Regression analysis was performed by plotting WS participant data points by age and PWV in those with, versus those without, diabetes (N=74, 7 with diabetes (black) and 67 without (gray)). Regression lines are shown. Neither the elevation nor the slope of the regression lines were significantly different (p=NS for both) in those with or without diabetes.

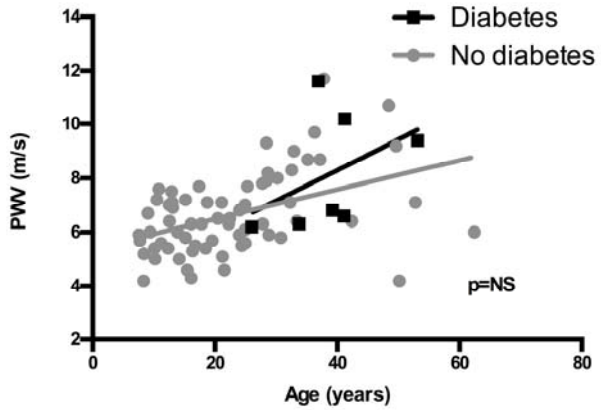

Figure S5. *NCF1* copy number modifies vascular disease severity in WS. Regression analysis is used to assess the effect of *NCF1* copy number on PWV (n=55). Removal of the single participant with the highest PWV (denoted # in Figure 3) increases the difference in PWV observed between CN=1 and CN=2 individuals, with those with *NCF1* CN≥2 (black) having higher PWV than those with CN=1 (gray) (p< 0.005 for elevation). In addition, there is a trend toward better protection from increasing PWV with older age (p value for slope improves from 0.6 (in Figure 3) to 0.1 (below).

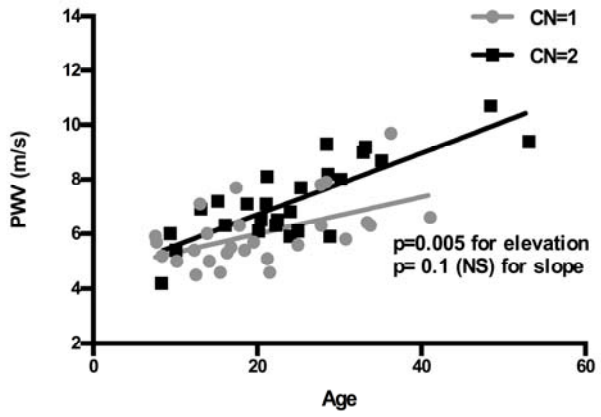

Figure S6. *NCF1* copy number modifies hypertension risk in WS. Fisher's exact test was performed to evaluate the prevalence of hypertension in WS participants with 1 *vs* ≥2 copies of *NCF1*. (N= 99 WS individuals for whom hypertension status and *NCF1* copy number were known). Gray bars show those with hypertension and black bars show those without. Prevalence of hypertension is reduced in those with *NCF1* CN=1 (p =0.03).