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Y chromosome *gr/gr* deletions are a risk factor for low semen quality

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BACKGROUND: Subfertility affects one in eight couples. In up to 50% of cases, the male partner has low semen quality. Four Y chromosome deletions, i.e. *Azoospermia factor a* (*AZFa*), *P5/proximal-P1* (*AZFb*), *P5/distal-P1* and *AZFc* deletions, are established causes of low semen quality. Whether a recently identified partial *AZFc* deletion, the *gr/gr* deletion, also causes low semen quality is at present unclear.

METHODS: We used a dual approach to review the effect of the *gr/gr* deletion on semen quality. First, we conducted a systematic review and meta-analysis of previous association studies, to compare the prevalence of *gr/gr* deletions between azoo-/oligozoospermic men and normozoospermic men. Secondly, we studied a cohort of 1041 male partners of subfertile couples unselected for semen quality. We employed a cross-sectional design by screening all men for the *gr/gr* deletion and comparing the semen quality of men with and without the *gr/gr* deletion.

RESULTS: Seven studies were included in the meta-analysis. The *gr/gr* deletion was significantly more prevalent among azoo-/oligozoospermic men than among normozoospermic men (OR 2.4, 95% CI 1.75–3.30). In our cohort, 25 men carried a *gr/gr* deletion. Men with this genotype had a lower sperm concentration (median 34×10^6/ml versus 53×10^6/ml, $P = 0.017$), total sperm count (median 108×10^6 versus 152×10^6, $P = 0.006$) and total motile sperm count (median 20×10^6 versus 50×10^6, $P = 0.010$) than men without the *gr/gr* deletion.

CONCLUSION: Y chromosome *gr/gr* deletions significantly reduce sperm counts and are thus associated with low semen quality.

Key words: Y chromosome / *gr/gr* deletion / spermatogenesis / semen quality / male infertility

Introduction

In the western world, 10–15% of couples suffer from subfertility, defined as the inability to conceive spontaneously after 1 year of unprotected intercourse. In up to 50% of couples the male partner is diagnosed with low semen quality (de Kretser, 1997; Evers, 2002).

Despite this high prevalence of low semen quality, reflected in the widespread use of ICSI (Centers for Disease Control and Prevention, 2005; Andersen *et al*., 2007), little is known about its etiology. Currently known causal factors, including hyperprolactinemia, hypogonadotrophic hypogonadism, previous chemo- or radiotherapy, bilateral cryptorchidism, congenital absence of, or surgery to, the vas deferens, orchitis, bilateral orchidectomy, structural and numerical chromosomal abnormalities and Y chromosome deletions, are only found in a minority of cases (de Kretser, 1997).

With respect to Y chromosome deletions, four recurrent deletions that cause azoo- or oligozoospermia have been identified to date: *Azoospermia factor a* (*AZFa*), *P5/proximal-P1* (*AZFb*), *P5/distal-P1* and *AZFc* deletions (Reijo *et al*., 1995; Vogt *et al*., 1996; Repping *et al*., 2002; Noordam and Repping, 2006). These deletions are present in 10–18% of European men with azoo- or oligozoospermia, and together they represent the most common known genetic cause of azoo- and oligozoospermia (Krausz and Degl'Innocenti, 2006).

Recently, we identified a novel recurrent Y chromosome deletion, termed the *gr/gr* deletion, that removes half of all genes in the *AZFc* region (Repping *et al*., 2003). The deletion was detected in 9 out of 246 men with azoo- or oligozoospermia and was absent in 148 men with normozoospermia. Findings in later studies were not as clear cut, with some studies confirming and others failing to confirm an association between the *gr/gr* deletion and azoo- or oligozoospermia (Fernandes *et al*., 2002; Machev *et al*., 2004; de Llanos *et al*., 2005; Ferlin *et al*., 2005; Giachini *et al*., 2005; Hucklenbroich *et al*., 2005; Lynch *et al*., 2005; Carvalho *et al*., 2006; de Carvalho *et al*., 2006; Ravel *et al*., 2006; Zhang *et al*., 2006).

In view of this inconsistency we aimed to review the association of the *gr/gr* deletion with low semen quality. To this end we took a dual

Table I Studies excluded from meta-analysis

First author	Year of publication	Reason for exclusion
Foresta	2005	Does not study *gr/gr* deletions, no STS PCR for *gr/gr*
Hadj-Kacem	2006	Does not study *gr/gr* deletions, no STS PCR for *gr/gr*
Mantas	2007	Does not study *gr/gr* deletions, no STS PCR for *gr/gr*
Mitra	2008	Does not study *gr/gr* deletions, no STS PCR for *gr/gr*
Simoni	2008	No association study on *gr/gr* and semen quality
Vogt	2005	No association study on *gr/gr* and semen quality
Writzl	2005	Does not study *gr/gr* deletions
Martinez-Garza	2008	Does not study *gr/gr* deletions, no STS PCR for *gr/gr*
Li	2008	No association study on *gr/gr* and semen quality
de Carvalho	2006	Sampling fertile/infertile males
Carvalho	2006	Sampling infertile males, fathers
Imken	2007	Fertile controls, fathers
Lardone	2007	Fertile controls
Lin	2007	Fertile controls
De Llanos	2005	Fathers as controls
Lynch	2005	Normozoospermic cases, fathers as controls
Machev	2004	Fathers as controls
Muslumanoglu	2005	No STS PCR for *gr/gr*
Ravel	2006	Fathers as controls
Stouffs	2008	Fertile controls
Wu	2007	Fathers as controls
Yang	2006	Fathers as controls
Zhang	2007	Fathers as controls
Ravel	2009	Fathers as controls
Lu	2009	Infertile normozoospermic men as cases
Yang	2006	Fathers as controls
Giachini	2005	Overlap with better or larger study
Yang	2008	Overlap with better or larger study

approach. First, we performed a systematic review and meta-analysis of previous case–control studies. Secondly, in a cross-sectional cohort design we studied a cohort of 1041 consecutively included male partners of subfertile couples unselected for semen quality. We genotyped all men for *gr/gr* deletions and compared semen parameters of men with and without the deletion.

Materials and Methods

Meta-analysis

For the meta-analysis, we followed the Human Genetic Epidemiology and Meta-analysis Of Observational Studies in Epidemiology guidelines (Stroup *et al*., 2000; Bray *et al*., 2007). We searched MEDLINE and EMBASE for all published studies on the effect of the *gr/gr* deletion on spermatogenesis with the following search strategy: (semen OR spermatogen*) AND (Y chromosome or *AZFc*) AND (*gr/gr* OR DAZ OR deletion), limited to articles on humans published in English from 1 October 2003 (Medline) or 2003 (EMBASE) to 1 January 2009. In addition, we scanned the reference list of relevant articles. This search was performed in duplicate by two independent reviewers (L.V. and S.R.). We only included association

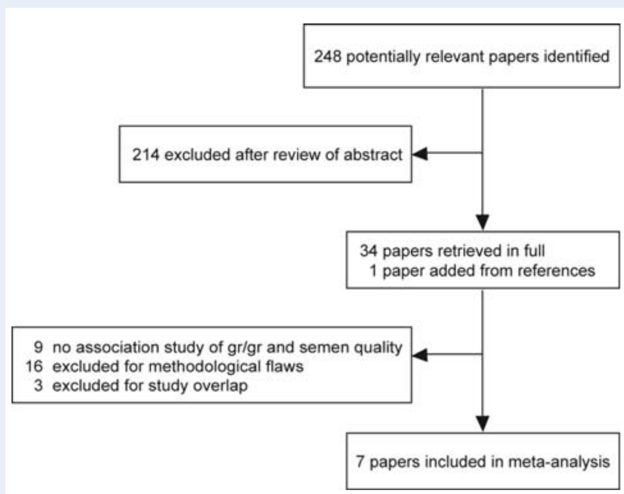

Figure 1 Study selection.

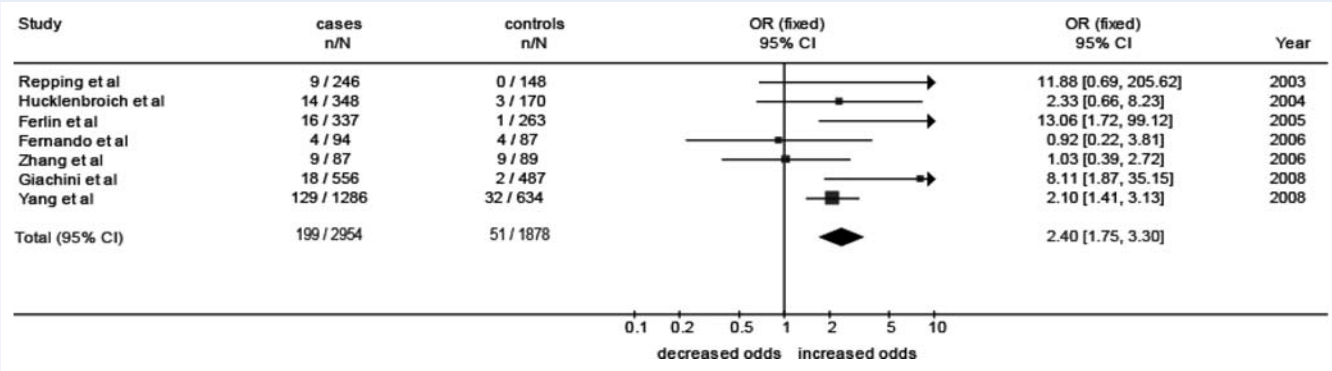

Figure 2 Meta-analysis of previous case–control studies.

Plotted are the individual and the total combined odds ratios with 95% confidence intervals. Odds ratios below one indicate that the *gr/gr* deletion is more common among controls, although odds ratios above one indicate that the *gr/gr* deletion is more common among cases. The combined Odds Ratio is 2.40 (95% CI 1.75–3.30). Test for heterogeneity: $\chi^2 = 11.66$, df = 6 ($P = 0.07$), $I^2 = 48.5\%$. Test for overall effect: $Z = 5.41$ ($P < 0.00001$).

Table II Patient characteristics

	Cohort (n = 1041)	Cohort without *gr/gr* deletions (n = 1016)	*gr/gr* deletions (n = 25)
Age (year)	36.7 ± 6.6	36.7 ± 6.6	34.0 ± 4.3
Country of origin			
Netherlands	681 (65)	669 (66)	12 (48)
Surinam and Dutch Antilles	103 (10)	100 (10)	3 (12)
Morocco	37 (4)	33 (4)	4 (16)
Turkey	30 (3)	28 (3)	2 (8)
Ghana	25 (2)	25 (2)	0
Other	141 (14)	137 (13)	4 (16)
Unknown	24 (2)	24 (2)	0
Semen quality[a]			
Volume (ml)	3.3 ± 1.5	3.3 ± 1.7	3.5 ± 1.6
Concentration (10^6/ml)	52 (16–89)	53 (16–90)	34 (4–65)
Motility (% progressive)	32 ± 16	30 ± 17	26 ± 18
Morphology (% normal)	37 (25–46)	37 (25–46)	32 (22–41)
Total count (10^6)	149 (44–281)	152 (45–284)	108 (5–171)
Total motile count (10^6)	49 (7–110)	50 (7–113)	20 (1–74)

Age and semen data are presented as mean ± SD or median (25th–75th percentile), ethnicity data as number (%).
[a]Based on 2495 semen analyses (on average 2.4 semen analyses per patient).

studies that defined the *gr/gr* deletion on the basis of sequence tagged site/polymerase chain reaction (STS/PCR) results as provided in the original report by Repping *et al*. (sY1291 negative, sY1161, sY1191, sY1206 and sY1201 all positive), which has been validated to provide the most robust analysis of deletions in the *AZFc* region, and included azoospermic and/or oligozoospermic men as cases and normozoospermic men as controls. When two or more studies from the same institution were identified, the study of larger size or better quality was selected. Data were extracted independently and in duplicate by two reviewers (L.V. and S.R.) using a standardized extraction form. These authors also independently assessed study quality in a standardized manner. Any disagreement was settled by a third author (F.V.).

Cohort analysis

We consecutively included male partners of subfertile couples presenting at the Center for Reproductive Medicine of the Academic Medical Center for a fertility workup, from January 2000 until July 2007. All men were included prior to semen analyses. Data on medical history and parents' and grandparents' country of origin were entered in a database at first visit. The study was approved by the Institutional Review Board of the Academic Medical Center. Written informed consent to store and use their DNA for research purposes was obtained from all men.

We excluded men with known causes of spermatogenic failure: i.e. hyperprolactinemia, hypogonadotrophic hypogonadism, previous chemo- or radiotherapy, bilateral cryptorchidism, congenital absence or surgery

Table III Semen parameters of men carrying a *gr/gr* deletion

Patient ID	Total count ($\times 10^6$)	Concentration ($\times 10^6$/ml)	Total motile count ($\times 10^6$)	Volume (ml)	Grade a motility (%)	Normal morphology (%)
01.0139.3	0	0	0	1.3		
01.0141.3	0	0	0	3.7		
AMC0387	0	0	0	2.7		
AMC0439	0	0	0	4.1		
AMC1026	0	0	0	5.2		
AMC0785	1	0	0	6.5	23	10
AMC1064	9	7	1	1.4	11	39
AMC0889	12	9	1	1.5	9	19
AMC0826	44	34	20	1.3	46	30
01.9008.3	55	8	6	6.5	12	38
01.2004.3	63	19	8	3.3	17	26
AMC0528	97	32	20	2.8	24	39
AMC1237	108	76	51	1.4	44	25
AMC0569	117	67	38	1.8	32	51
AMC0817	123	24	13	5.1	13	22
AMC0372	133	37	59	3.6	44	41
AMC1285	150	50	63	3.0	43	19
AMC0524	155	58	75	2.3	35	54
AMC1057	158	63	10	2.4	5	50
AMC1044	184	59	73	3.2	40	60
AMC1016	195	98	81	2.0	42	25
AMC0655	201	105	106	1.9	53	35
AMC1260	224	53	96	4.1	39	32
AMC0530	270	83	82	3.3	31	8
AMC0802	321	83	106	3.9	33	10

of the vas deferens, orchitis, and bilateral orchidectomy. Men were also excluded if the fertility workup identified retrograde ejaculation, obstructive azoospermia, an *AFZa*, *P5/proximal-P1*, *P5/distal-P1* or *AZFc* deletion, or numerical or structural chromosome abnormalities. Grade 2/3 varicoceles or recurrent infections were not routinely screened for as part of the fertility workup.

For each patient at least two semen analyses were performed according to WHO guidelines (World Health Organization, 1999) and retrospectively linked to each included patient.

Deletion screening

Genomic DNA was extracted from peripheral blood leukocytes collected from a venous blood sample. Deletion screening was performed using six plus/minus PCR assays (sY142, sY1197, sY1191, sY1291, sY1206, sY1201) as described previously (Repping *et al*., 2003). All STS–PCRs were performed in a simplex reaction with factor VIII as an internal control, thus reported deletions are considered to be true deletions.

Statistical analysis

We used Cochrane Collaboration's RevMan 4.2 software (http://www.cc-ims.net/RevMan) to perform the meta-analysis. Heterogeneity was assessed by calculation of the I^2 statistic. Meta-analysis of odds ratios was performed using a fixed-effects model.

In the cohort study, we used the average of all available semen analyses of each patient in all statistics. Semen quality of both genotype groups was compared using either *T*-tests for normally distributed, or Mann–Whitney tests for non-normally distributed semen parameters. Data were tested one-sidedly since our hypothesis was that *gr/gr* deletions would decrease and not increase semen quality. Analyses were carried out using the statistical package SPSS for Windows 14.0.2.

The *R* code used to plot the distributions of total sperm counts for men with and without the *gr/gr* deletion and to estimate the smoothed distributions is available on request. Briefly, we used the *R* 'density' function with a Gaussian kernel of bandwidth 80.8 applied to the total sperm count data after reflecting it around 0 to avoid discontinuity at a total sperm count of 0 (see Section 5.6, Density Estimation; Venables and Ripley, 1999). We selected 80.8 as the mean of the bandwidths chosen by the 'width.SJ' function (*R* library MASS) for the two distributions of total sperm counts. *R* is available at http://r-project.org.

Results

Systematic review and meta-analysis

Our search returned 209 studies in EMBASE and 157 in Medline, of which 118 were found in both. Thus, abstracts of 248 unique studies were reviewed. We directly discarded 62 articles based on the publication type (reviews, case reports and other non-original

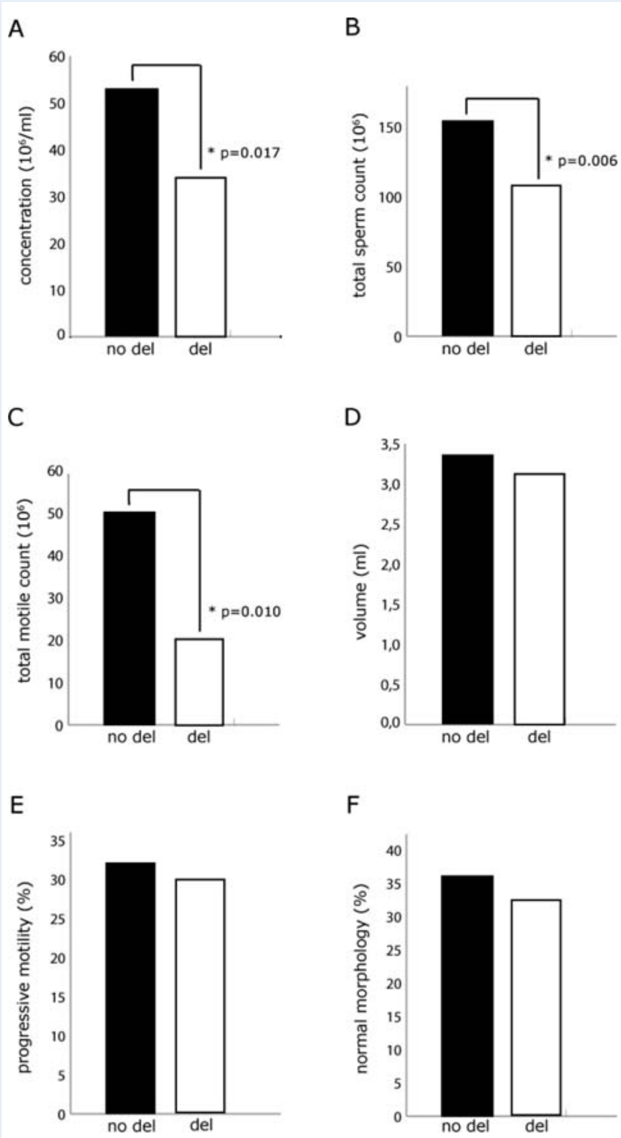

Figure 3 Comparison of semen parameters of men with and without a *gr/gr* deletion.

Comparison of median parameters for concentration (**A**), total sperm count (**B**), total motile count (**C**) and comparison of mean values for semen volume (**D**), motility (**E**) and morphology (**F**). * Indicates statistical significance.

research articles) and 125 studies that clearly were not association studies into the effects of *gr/gr* deletions on spermatogenesis. In addition, the EMBASE search included articles published before October 2003 ($n = 27$), that were also discarded. Next, 34 articles were retrieved in full. Of these, nine articles were not original research articles on the association of the *gr/gr* deletion with semen quality. Hand-searching of the reference lists of all retrieved articles returned one additional article that was retrieved in full. Thus, a total of 26 studies reported on the association of the *gr/gr* deletion with semen quality. Of these, 16 articles were excluded because of methodological issues, i.e. including men with unknown semen quality or using inappropriate methods to identify *gr/gr* deletions. Three studies showed overlap with another study from the same institution. All studies that were excluded from the meta-analysis and the reason for their exclusion are shown in Table I. Seven studies remained for meta-analysis (Fig. 1). The I^2 statistic (48.5%, $P = 0.09$) indicated moderate statistical heterogeneity between the studies. Combined, 199 of 2954 azoo- or oligozoospermic men (6.7%) had a *gr/gr* deletion versus 51 of 1878 normozoospermic men (2.7%). (OR 2.40, 95% CI 1.75–3.30) (Fig. 2).

Additionally, we conducted a secondary meta-analysis including all azoospermic and oligozoospermic cases and normozoospermic controls that could be extracted from the studies that also sampled normozoospermic cases and 'fertile' controls. Combined then, 277 of 4072 azoo- or oligozoospermic men (6.8%) had a *gr/gr* deletion versus 97 of 2426 normozoospermic men (4.0%). This secondary analysis returned a lower OR (or 1.87, 95% CI 1.46–2.38), most likely because of increased heterogeneity (I^2 62.2%, $P = 0.002$).

Cohort study

We consecutively included 1041 men. Baseline characteristics of these men including semen parameters are summarized in Table II. The *gr/gr* deletion was found in 25 men (2.4%). Age and country of origin of the *gr/gr* deleted men did not differ from that of the undeleted cohort. The semen parameters of the *gr/gr* deleted men are shown in Table III.

Men with the *gr/gr* deleted genotype had significantly lower sperm concentrations (median 34×10^6/ml versus 53×10^6/ml, $P = 0.017$), total sperm counts (median 108×10^6 versus 152×10^6, $P = 0.006$) and total motile sperm counts (median 20×10^6 versus 50×10^6, $P = 0.010$) than men without the deletion (Fig. 3). Semen volume, sperm motility and sperm morphology were not significantly different.

The distributions of the total sperm counts of men with and without *gr/gr* deletions are plotted in Fig. 4. The distribution varied from 0 to 972×10^6 in men without a deletion and from 0 to 321×10^6 in men with a *gr/gr* deletion.

Discussion

Our meta-analysis of seven case–control studies showed that *gr/gr* deletions were more prevalent in men with azoo- or oligozoospermia. In addition, the cohort study of 1041 men unselected for sperm counts showed that men with *gr/gr* deletions had significantly lower sperm counts. Taken together these data indicate that the *gr/gr* deletion is associated with low semen quality.

More than half of previous case–control studies were excluded from meta-analysis, because they employed fertility-based sampling, i.e. they used 'proven fertile men' or 'fathers' as controls. Fertility, however, is a characteristic of the couple, which in turn is heavily dependent on the characteristics of the female partner. It is well known that even severely oligozoospermic men can father children naturally as long as they have a fertile partner. Thus, 'normozoospermic' and 'fertile' are not synonymous.

Statistical testing for heterogeneity in the meta-analysis returned an I^2 of 48.5%, indicating that 48.5% of variation across studies is due to heterogeneity rather than chance (Higgins *et al.*, 2003). A differential effect of the *gr/gr* deletion in European compared with Asian populations may be a source of heterogeneity. In the meta-analysis, studies in Asian

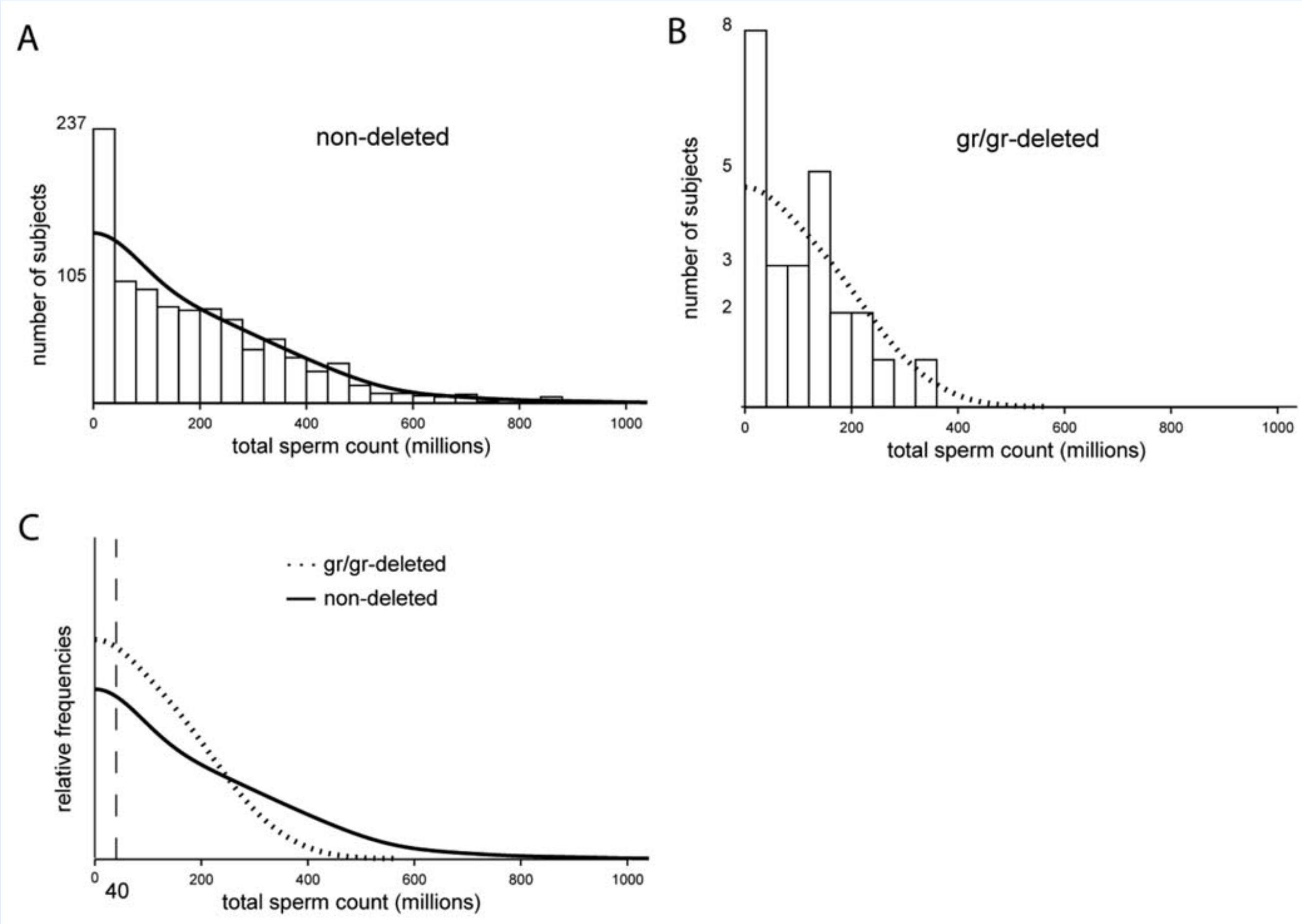

Figure 4 Total sperm count distributions.

Distribution of total sperm counts for (**A**) men without detectable Y chromosome deletions and (**B**) for men with *gr/gr* deletions. The distribution in (B) is significantly lower than that in (A) ($P = 0.006$, Wilcoxon rank sum test, one sided). Lines representing the smoothed distributions are superimposed over the histograms in panels A and B (see Materials and Methods). (**C**) The two smoothed distributions superimposed. The dashed line indicates the WHO cut-off value for normozoospermia that was used in previous studies that included azoo- or oligozoospermic cases and normozoospermic controls.

populations report higher frequencies of *gr/gr* deletions in both cases and controls compared with Europeans (prevalence in all Asian men taken together 8.3% compared with 2.5% in all European men combined). Separate pooling of Asian and European studies reduces I^2 to 26.0 and 4.7%, respectively, and results in an OR of 1.84 (96% CI 1.29–2.16) for Asian studies and an OR of 6.17 (96% CI 2.75–13.84) for European studies (data not shown). Genetic or environmental factors underlying these differences are currently unknown.

When we identified the *gr/gr* deletion in 2003, we hypothesized that secondary duplications in *gr/gr* deleted chromosomes might act as a compensatory mutation by restoring gene copy number (Repping *et al*., 2003). Gene dosage analysis to search for such duplications is not routinely performed, however, and only a few of the studies included in the meta-analysis have investigated *AZFc* gene copy number. Moreover, a recent study in Europeans showed no compensatory effect of a secondary duplication following a *gr/gr* deletion (Krausz *et al*., 2009).

Previous studies on the association between *gr/gr* and semen quality all used a case–control design. In fact, case–control designs have been frequently used to study the association of genetic or environmental factors with spermatogenesis (Bonde *et al*., 1996; Gianotten *et al*., 2004). However, this design has significant disadvantages. Most importantly, it requires a phenotypic dichotomization into cases and controls. As spermatogenesis is a quantitative, continuous trait, dichotomous analysis is far from ideal. The phenotypic effect imparted by a single locus is small and may go undetected using a coarse and biologically arbitrary division in cases and controls. A cross-sectional cohort design on the other hand is well suited to examine phenotypic variability. In other research fields where the association between genetic variants and quantitative traits is studied, this design is already often used. Examples include the study of associations between genetic polymorphisms and bone mass or atherosclerosis markers (Richert *et al*., 2007; Samani *et al*., 2008). We argue that in the field of male infertility, examining genotype-phenotype associations in large cohorts of men with variable sperm counts is the optimal way to identify genetic factors associated with low semen quality.

Our finding that the *gr/gr* deletion acts as a risk factor for low semen quality and hence does not always lead to azoo- or oligozoospermia, stands in contrast to the effects of *AZFa*, *P5/proximal-P1* (*AZFb*), *P5/distal-P1* and *AZFc* deletions that always cause azoo- or severe oligozoospermia. It is yet unknown which additional, genetic or environmental, factors modulate the phenotypic effect of *gr/gr* deletions.

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