Androgen receptor CAG polymorphism (Xq11-12) status and human spermatogenesis: A prospective analysis of infertile males and their offspring conceived by intracytoplasmic sperm injection

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Abstract. We determined the association of androgen receptor (AR) (CAG) n lengths among fertile and infertile males and offspring conceived by intracytoplasmic sperm injection (ICSI). Assessment of (CAG)n repeats in the AR was performed in a Caucasian population by gene sequencing in fertile men (n=13), infertile men (n=64), boys conceived after ICSI (n=21), and boys conceived naturally (n=11). In the AZF region of the Y chromosome, a total of 22 STSs were analyzed by multiplex PCR; selected spermatozoa were also analyzed by fluorescent in situ hybridization (FISH) for chromosomes 18, 21, X, and Y. The average age was 43.7±7 yr for infertile, 44.8±7 yr for fertile men, and 5.0±0.5 yr for the children. The mean (CAG)n was 22.2±3 for the infertile men and 19.3±5 for fertile controls. There was a significant difference in CAG repeat length in the severely oligo-/azoospermic men vs. controls (p=0.02). An inverse correlation was evident between CAG length and semen parameters. For ICSI male children, the AR (CAG)n lengths were 21.4±3.2 vs. 20.8±3.4 for boys conceived naturally. While all peripheral karyotypes of fertile and infertile men were normal, de novo gonosomal abnormalities were observed in the ICSI offspring. The incidence of Y microdeletions was 1.6% in infertile men; all the ICSI sons had an intact Y chromosome. In conclusion, severely oligo- and azoospermic men had longer CAG repeat length than fertile controls, suggesting that certain AR gene mutations may have a negative effect on spermatogenesis. An increased incidence of de novo gonosomal abnormalities was found in the ICSI offspring when compared to children conceived naturally. Our assessment of the polymorphic region of the AR gene, in the absence of other specific genomic abnormalities, suggests that the fertility of children conceived by ICSI may be conserved.

Introduction

Intracytoplasmic sperm injection (ICSI) potentiates successful treatment of infertile couples with in vitro fertilization where the male partner has compromised sperm parameters (1,2). Many of these infertile men have impaired spermatogenesis and often exhibit related genetic disorders including chromosomal aneuploidy, structural chromosomal rearrangements, or mutations/deletions that are assessable only at the molecular level (3). Even when chromosomal defects are not observed in the peripheral karyotype, in spermatozoon these are about twice as common as in normal donor semen (4,5). For example, single gene mutations such as those on chromosome 7 and deletions in the Yq AZF may be present in men with obstructive azoospermia and carriers of the cystic fibrosis trait (6,7).

Both spermatogenesis and normal male sexual development are under the control of the androgen receptor (AR) gene, located on the long arm of the X chromosome at Xq11-12 (8). The AR gene has 8 exons that encode 3 protein domains. A polymorphic CAG (glutamine) repeat sequence is in exon 1. The AR-CAG repeat region is unstable and its length may sometimes undergo expansion or contraction during meiotic DNA replication. Mutations in this gene have been associated with various disorders including complete androgen insensitivity syndrome (9), various motor neuron diseases, and prostate and ovarian cancers (10). The length of CAG repeats influences AR function; enhanced androgen action is associated with fewer repeat lengths and decreased AR function occurs with longer CAG repeat lengths. Interestingly, the AR also plays an important role in the nervous system. Males with Kennedy's disease (a serious
neurodegenerative disorder) exhibit over 40 CAG repeats in the AR gene and show compromised spermatogenesis and subsequent infertility (11). Normally, CAG repeat length is between 9 and 36 (12) although an even narrower range (8 to 27) has been detected in some fertile men (13,14). The length of these (CAG)$_n$ has also been noted to vary by ethnicity, with an average of 21 repeats in Caucasian men, 18 in African-Americans, and 22 in Asian men (15).

This study investigated the relationship between fluctuations in the trinucleotide repeat (TNR) region and semen characteristics of Caucasian infertile men treated by ICSI. A group matched for age and ethnicity was used as a control. We also assessed the CAG repeat length in male children conceived by ICSI and compared them to boys from natural conception. To identify any other factors affecting spermatogenesis, we conducted additional genetic tests for male infertility such as Yq microdeletion and chromosomal analysis of spermatozoa.

Again this background, the study aimed to screen infertile men and their children for any relationship between spermatogenesis and their genomic profile. We also investigated whether sequencing of the AR gene may predict subsequent spermatogenesis in ICSI offspring.

Materials and methods

Patients. Following institutional review board approval (IRB #0299-581), a total of 64 Caucasian males with abnormal sperm parameters and 21 ICSI sons provided blood samples for genetic characterization. Control subjects consisted of 13 fertile males matched for age and ethnicity as well as 11 boys conceived without medical assistance.

Specimen classification. For this investigation, semen parameters were considered normal when a concentration of ≥20x10$^6$/ml, a progressive motility of ≥40%, and a normal morphology of ≥4% were confirmed. Sperm concentrations were stratified as follows: mild oligospermia (≥5 to <20x10$^6$/ml), moderate oligospermia (1 to <5x10$^6$/ml), severe oligospermia (<1x10$^6$/ml), or azoospermia (zero spermatozoa).

Extraction of genomic DNA. DNA was purified from peripheral leukocytes with the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), as previously described (16). DNA content was quantified by optical density determination; DNA concentration/quality were also estimated in relation to control high molecular weight DNA processed by peripheral leukocytes with the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). Reactions were thermo-cycled in a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems). Purified products were electrophoresed on a SpectruMedix 9610 Aurora™ DNA Sequencer (SpectruMedix LLC, State College, PA, USA) (Fig. 1).

DNA sequencing of AR gene. PCR product was purified by the QIAquick PCR purification kit (Qiagen). DNA sequencing reactions were assembled with ABI PRISM® BigDye™ Terminators V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Reactions were thermo-cycled in a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems). Purified products were electrophoresed on a SpectruMedix 9610 Aurora™ DNA Sequencer (SpectruMedix LLC, State College, PA, USA) (Fig. 1).

Where biallelic analysis of specimens was undertaken, DNA templates amplified with fluorescent-labeled primers were processed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem) for fluorescent quantification. The (CAG)$_n$ was derived by subtracting a constant non-repetitive residue (in bp number) from the total number of bases of the synthesized amplicon in the exon 1 region of the AR gene for each X chromosome. The constant residue was measured by performing the analysis in a karyotypically normal man where the (CAG)$_n$ was assessed by sequencing the AR gene motif and subtracting it from the total bp number obtained by quantification analysis.

Additional genetic studies. Chromosome analysis was performed on phytohemaglutinin (PHA)-stimulated peripheral blood lymphocytes with G-band staining. At least 20 metaphase cells were counted and a minimum of 5 cells was analyzed. In cases of complex structural chromosome aberrations, additional analysis by fluorescent in situ hybridization (FISH) was performed. All patients diagnosed with a chromosomal abnormality were offered genetic counseling.

To assess for Y chromosome microdeletions in the azoospermia factor (AZF) region, a total of 22 sequence tag sites (STSs) were analyzed by multiplex PCR and deletion breakpoints were defined with additional loci (16). Sperm cell aneuploidy screening was performed for chromosomes 18, 21, X, and Y by FISH analysis (4,5).

Data analysis. Statistical analysis was carried out by independent sample t-test to evaluate all mean comparison hypotheses. A logistic regression model was evaluated for fertility status as a function of CAG repeats. The odds ratio estimate associated with fertility status yielded a point estimate of 1.28 with 95% Wald confidence limits (1.05-1.57). For evaluation of the hypothesis of an increase in chromosomal abnormalities as sperm count decreases, a test
of linear trend of proportions was conducted. Two-tailed tests were utilized and only p<0.05 was considered significant. Statistical differences are reported in text and tables only when significant.

Results

Infertile males. Study subjects (n=77) provided one sample each and these were classified as azoospermic (n=7), oligozoospermic (n=43), asthenozoospermic (n=40), and teratozoospermic (n=59). Normozoospermic males of proven fertility (n=13) served as controls. Mean (± SD) age of infertile subjects and controls was 43.7±7 and 44.8±7 yr, respectively. The average length of (CAG)n repeats in the infertile men was significantly higher than the control group (22.2±3 vs. 19.3±5; p=0.02 by t-test). When logistic regression was performed using CAG repeats, the odds ratio for the fertility status was 1.28 (95% CI: 1.05-1.57) (p=0.01) (Table II). When the

Table I. Primer sequences used to synthesize exon 1 of the human androgen receptor gene.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Sequence</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoallelic</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5'- TTC AGA ATC TGT TCC AGA GCG TGC -3'</td>
<td>279</td>
</tr>
<tr>
<td>R</td>
<td>5'- GCT GTG AAG GTT GCT GTT CCT CAT -3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biallelic</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5'- 6fam-TTC AGA ATC TGT TCC AGA GCG TGC -3'</td>
<td>279</td>
</tr>
<tr>
<td>R</td>
<td>5'- GCT GTG AAG GTT GCT GTT CCT CAT -3'</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
repeat lengths were ranked by semen characteristics, an inverse relationship was observed between the microsatellite sequence and sperm concentration and morphology (p=0.02 and p=0.04, respectively) (Tables III and IV), but not with sperm motility. Plotting the proportion of infertile individuals according to their repeat lengths versus sperm characteristics, a repeat length of less than (CAG)17 was consistently associated with normal concentration (Fig. 2), motility, and morphology (Fig. 3). In contrast, microsatellite sequences over 25 in number correlated consistently with compromised concentration (Fig. 2) and morphology (Fig. 3), but not with sperm motility.

A normal peripheral karyotype was confirmed for all study subjects and controls. Yq deletion was identified in one patient (1.3%) with an AZFc deletion. This subject also had the longest length of (CAG)32. Chromosomal status was assessed by FISH analysis for 30 semen samples, revealing an overall incidence of chromosomal abnormalities of 1.5% (Table V). For all abnormality categories (autosomal, gono-

### Table II. Observed CAG polymorphism lengths of ICSI and control (fertile) males.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>CAG repeats (mean ± SD)</th>
<th>Range</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICSI</td>
<td>64</td>
<td>22.2±3a</td>
<td>17-32</td>
<td>21.5-22.9</td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>19.3±5a</td>
<td>10-24</td>
<td>15.6-21.0</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>21.7±4</td>
<td>10-32</td>
<td>21.0-22.4</td>
</tr>
</tbody>
</table>

CI, confidence interval; ICSI, intracytoplasmic sperm injection; *by Student’s t-test (two independent samples), effect of CAGₙ polymorphism length on fertility status, p=0.02.

### Table III. The relationship between human androgen receptor CAG polymorphism lengths and sperm concentration.

<table>
<thead>
<tr>
<th>Sperm concentration (x10⁶/ml)</th>
<th>n</th>
<th>CAG repeats (mean ± SD)</th>
<th>Range</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>13</td>
<td>23.1±4a</td>
<td>19-32</td>
<td>21.5-24.7</td>
</tr>
<tr>
<td>1-&lt;5</td>
<td>14</td>
<td>22.8±4a</td>
<td>17-30</td>
<td>21.2-24.4</td>
</tr>
<tr>
<td>5-&lt;20</td>
<td>16</td>
<td>21.6±2</td>
<td>18-26</td>
<td>20.7-22.6</td>
</tr>
<tr>
<td>≥20</td>
<td>34</td>
<td>20.6±3b</td>
<td>10-25</td>
<td>19.6-22.4</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>21.7±4</td>
<td>10-32</td>
<td>21.0-22.4</td>
</tr>
</tbody>
</table>

CI, confidence interval; * vs b, t-test using ≥20x10⁶/ml concentration as reference: effect of sperm concentration on CAG polymorphism length, p<0.02.

### Table IV. The relationship between human androgen receptor CAG polymorphism lengths and sperm morphology.

<table>
<thead>
<tr>
<th>% normal morphology</th>
<th>n</th>
<th>CAG repeats (mean ± SD)</th>
<th>Range</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>33</td>
<td>22.5±3a</td>
<td>18-32</td>
<td>21.5-23.5</td>
</tr>
<tr>
<td>1-&lt;4</td>
<td>26</td>
<td>21.8±3</td>
<td>17-30</td>
<td>20.7-22.9</td>
</tr>
<tr>
<td>≥4</td>
<td>18</td>
<td>19.9±4b</td>
<td>10-25</td>
<td>18.1-21.7</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>21.7±4</td>
<td>10-32</td>
<td>21.0-22.4</td>
</tr>
</tbody>
</table>

CI, confidence interval; * vs b, Student’s t-test (two independent samples): effect of sperm morphology on CAGₙ length, p<0.04.
been demonstrated by several investigators (13,19-24). In this polymorphic trinucleotide (CAG) repeat in the AR gene has defects to the offspring.

about the possibility of vertical transmission of genetic CF mutations). Such genetic derangements support concern microgenomic defects (i.e., Y chromosome microdeletions, study of the genetics of male infertility. Such genetic advanced reproductive technologies has attracted closer Discussion

naturally conceived offspring. (Table VII). No AZF deletions were identified in the ICSI or range for a fertile population of a similar ethnic background

normal karyotypes. The CAG repeat lengths of ICSI and 24 repeats (Table VI). All three fathers of these children had a homozygous 21 repeat length, and the 47, XYY case had Klinefelter case had (CAG)19/21, the mosaic Klinefelter case had (Fig. 4B), and a third 47, XYY. The complete Klinefelter case had (CAG)19/21, the mosaic Klinefelter case had a homozygous 21 repeat length, and the 47, XYY case had 24 repeats (Table VI). All three fathers of these children had normal karyotypes. The CAG repeat lengths of ICSI and spontaneously conceived children were within the expected range for a fertile population of a similar ethnic background (Table VII). No AZF deletions were identified in the ICSI or naturally conceived offspring.

ICSI male offspring. A genetic characterization was undertaken for 21 ICSI boys and 11 naturally conceived sons (controls). These children had an average age of 5±0.5 yr. The mean AR-CAG length among controls was 20.8±3.4 and 21.4±3.2 for the ICSI offspring (Table VI). Three boys conceived by ICSI had abnormal karyotypes: one a 47, XXY (complete Klinefelter) (Fig. 4A), another was a 46, XY/47, XXY (mosaic Klinefelter) (Fig. 4B), and a third 47, XYY. The complete Klinefelter case had (CAG)19/21, the mosaic Klinefelter case had a homozygous 21 repeat length, and the 47, XYY case had 24 repeats (Table VI). All three fathers of these children had normal karyotypes. The CAG repeat lengths of ICSI and spontaneously conceived children were within the expected range for a fertile population of a similar ethnic background (Table VII). No AZF deletions were identified in the ICSI or naturally conceived offspring.

Discussion

The integration of ICSI as a central component of the advanced reproductive technologies has attracted closer study of the genetics of male infertility. Such genetic abnormalities can manifest at a macrogenomic level (i.e., entire structural chromosomal aberrations) or be limited to microgenomic defects (i.e., Y chromosome microdeletions, CF mutations). Such genetic derangements support concern about the possibility of vertical transmission of genetic defects to the offspring.

A link between male infertility and expansion of the polymorphic trinucleotide (CAG) repeat in the AR gene has been demonstrated by several investigators (13,19-24). In this study, we confirmed the inverse relationship between the polymorphic region of the AR gene and sperm production, specifically in regard to concentration and morphology. Our observations may appear to disagree with prior studies which failed to identify such a relationship (25-32) or at variance with work that focused on a single semen parameter (33). However, because the association between the AR gene and sperm production are ethnically variable, important trends may be difficult to identify in a heterogeneous population. When patients analyzed in the present study were stratified according to CAG lengths, the large majority of the AR polymorphic segments ranged between 18 and 24 repeats. Below (CAG)17, semen production was preserved. Thus, trinucleotide repeats above (CAG)26 indicate compromised concentration and morphology, though with only minor effects on motility.

Data from the present study corroborate findings of previous reports, where fertile males with short CAG repeat sequence had the highest sperm output (34), and subjects with ≥26 AR-CAG repeats being at more than 4-fold increased risk for impaired spermatogenesis (35). We found males with an infertility problem were more likely than controls to have an expanded polymorphic region of the AR gene. Confirmation of the link between the expanded CAG sequence and spermatogenic defects in Caucasian men is parallel to similar studies in Asian, Indian, Australian, and North American populations (20,27,36,37). Moreover, Eckardstein et al (38) reported Caucasian men with incomplete gonadotrophin suppression were 2.5 times more likely to be azoospermic when an expanded CAG sequence was present.

Instability of the AR-CAG sequence may arise via slipped mispairing at the replication fork, allowing an insertion when a newly synthesized strand dissociates from the primer strand during replication synthesis (39,40). The new strand may then align with the repeat copy rather than its cognate copy. It has been observed that longer repeats are identifiable also in kidney cells kept in culture for several months (40), but in this study the (CAG)n of ICSI sons was within the expected range, indicating that the in vitro process did not induce expansion of the trinucleotide sequence.

Although the AR gene (X chromosome) is not transmitted paternally to male offspring, it does directly pass to female offspring with a 95% chance of identical length (22). Thus

Table V. Fluorescent in situ hybridization analysis of sperm related to sperm concentration.

<table>
<thead>
<tr>
<th>Sperm concentration (x10^6/ml)</th>
<th>n</th>
<th>Cells assessed</th>
<th>Autosomal%</th>
<th>Gonosomal%</th>
<th>Concurrent%</th>
<th>Total%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>4</td>
<td>580</td>
<td>12 (2.1)</td>
<td>20 (3.4)</td>
<td>4 (0.7)</td>
<td>36 (6.2)</td>
</tr>
<tr>
<td>1-&lt;5</td>
<td>1</td>
<td>1,008</td>
<td>30 (3.0)</td>
<td>11 (1.1)</td>
<td>7 (0.7)</td>
<td>48 (4.8)</td>
</tr>
<tr>
<td>5-&lt;20</td>
<td>4</td>
<td>7,827</td>
<td>30 (0.4)</td>
<td>20 (0.3)</td>
<td>8 (0.1)</td>
<td>58 (0.7)</td>
</tr>
<tr>
<td>≥20</td>
<td>21</td>
<td>59,049</td>
<td>323 (0.5)</td>
<td>329 (0.6)</td>
<td>209 (0.3)</td>
<td>861 (1.5)</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>68,464</td>
<td>395 (0.6)</td>
<td>380 (0.6)</td>
<td>228 (0.3)</td>
<td>1,003 (1.5)</td>
</tr>
</tbody>
</table>

*Test of linear trend in proportions across sperm concentration categories and among types of anomalies, p<0.05.

Table VI. CAG lengths of ICSI offspring with gonosomal abnormalities.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Karyotype</th>
<th>CAG repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46, XY/47, XXY</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>47, XXY</td>
<td>19/21</td>
</tr>
<tr>
<td>3</td>
<td>47, XYY</td>
<td>24</td>
</tr>
</tbody>
</table>

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the latter would be carriers (first generation), with their male offspring (second generation) having an estimated 50% chance of inheriting the mutation of the polymorphic region. However, in such carrier daughters there is a 5% risk that the CAG trinucleotide length may contract or expand by up to 8 unit base pairs, with consequent deleterious effects (22,41). This would apply also in the case of male ICSI offspring.

In screening ICSI offspring fathered by men with genetically abnormal sperm, it is necessary to assess other genetic disorders that could contribute to impaired spermatogenesis. While no Yq deletions were detected in our population, de novo gonosomal abnormalities were observed in ICSI children and such abnormalities can have a direct impact on fertility (42). For example, Klinefelter syndrome is characterized by a

Figure 3. Distribution of CAG repeat sizes within the AR gene of fertile and infertile men, as a function of sperm morphology. The green bars represent percentages (%) of men with normal sperm morphology (≥4%), and pink bars are used for teratozoospermic men (<4%). CAGs repeats <17 predicted spermatozoa with normal morphology, and those >26 predicted some degree of teratozoospermia.

Figure 4. GeneMapper profiles of androgen receptor (AR) alleles from two ICSI boys. (A) Complete Klinefelter form with heterozygocity (2 signals, blue) and (B), mosaic Klinefelter with homozygous X chromosomes (1 signal, blue).
potentially compromised spermatogenesis (43,44). However, the two Klinefelter children analyzed in our study both had a normal range of (CAG)n, suggesting spermatogenetic function may be conserved (45).

Knowledge of these genetic issues is essential for appropriate patient counseling and the development, where possible, of rational therapeutic plans. Outcome monitoring in the advanced reproductive technologies is critical, particularly regarding the future reproductive capacity of children conceived by ICSI (46-48). The present analysis suggests the application of ICSI has no bearing on the length of the polymorphic region of the AR gene in such male offspring.

Our findings also validate the concept that modulation of AR-CAG length directs spermatogenesis primarily as a secondary regulator of AR function. CAG repeats probably do not have a major independent effect on testis function, but rather may modify or fine-tune relevant endocrine feedback systems and hormone action. If this were not so, it would be expected that evolution selection pressure would cause such polymorphism to disappear from the reproductive pool (33). Thus, the overall fertility status of an individual would appear to depend not just on AR sequence alterations but also on interactions between other genomic and environmental factors. This multifactorial model implies that comparisons of reproductive potential based solely on sperm parameters may be insufficient to yield the complete picture, with consideration of the nuances the AR-CAG repeat yielding a more precise assessment.

Although the occurrence of de novo chromosomal abnormalities can be attributed to a mosaicism present in the sperm population of individuals with severe male infertility, a modification of the technique for biallelic analysis of the polymorphic region of exon 1 did allow measurement of the trinucleotide repeat length. It has been postulated that the AR-CAG is not linked to spermatogenesis in these individuals (45). However, mosaic or complete trisomic forms may be directly implicated in the compromised spermatogenesis (49), or, if maintained (45,50), spermatozoa produced would have a disproportionately higher number of chromosomal aberrations (5,51,52). This mosaic issue of the gamete may be insufficient to yield the complete picture, with consideration of the nuances the AR-CAG repeat yielding a more precise assessment.

Acknowledgments

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References


Table VII. Comparison of CAG polymorphism lengths between ICSI offspring and naturally conceived boys.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>CAG repeats</th>
<th>Range</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICSI</td>
<td>21</td>
<td>21.4 ± 3.2</td>
<td>13-26</td>
<td>20.0-22.8</td>
</tr>
<tr>
<td>Naturally conceived</td>
<td>11</td>
<td>20.8 ± 3.4</td>
<td>13-25</td>
<td>19.7-21.9</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>21.2 ± 3.2</td>
<td>13-26</td>
<td>20.6-21.8</td>
</tr>
</tbody>
</table>

CI, confidence interval.


