Sperm Ribosomal DNA: Biomarkers for Male Factor Infertility

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Abstract

In the United States, 15% of heterosexual couples are affected by clinical infertility. Male factor infertility is the sole or contributing factor in approximately 50% of these couples, affecting approximately 7% of men. Data compiled via retroactive studies and meta-analysis suggest decreasing semen count and quality over time and across generations in specific geographic regions and indicate effects by environmental exposures during the preconception spermiogenesis period. Previous investigation by the Environmental Epigenetics Lab has identified biomarkers for male factor infertility in mitochondrial DNA copy number (mtDNAcn), verifying mtDNAcn variation as a diagnostic tool for male fertility and pregnancy outcomes. These findings, coupled with a study from China linking mtDNAcn with ribosomal DNA copy number (rDNAcn) in relation to gastric tumor cells suggest that rDNAcn may be another viable indicator of infertility. The objective of this study is to investigate the relationship between sperm rDNAcn and semen parameters. Semen samples (n=140) from individuals undergoing assisted reproductive technology (ART) in the Sperm Environmental Epigenetics Development Study (SEEDS) at Baystate Medical Center in Springfield, MA were collected, and their semen parameters were assessed. A triplex probe-based qPCR approach was used to obtain copy numbers of the 5S and 18S rDNA subunits. Statistical analysis revealed an inverse association between copy number in the 18S

subunit ribosomal subunit and semen parameters, with moderately strong relationship and strong statistical significance.

Introduction and Review of Literature

As defined by the World Health Organization (WHO), clinical infertility is inability for sexually active heterosexual couples to achieve pregnancy in 12 months (Olayemi 2010, as cited in Puris and Christiansen, 1992). Infertility affects 15% of couples and is impacted by contributions by both female and male reproductive health. Maternal reproductive health studies - especially in the case of epidemiological impacts - have proven a popular avenue for research in reproductive health; despite this, male contribution to infertility is present in 50% of cases (Jungwirth et al., 2018). Male infertility can arise from irregularities in semen production which result in low sperm counts or decreased motility of sperm cells (Olayemi, 2010 as cited in Sinclair, 2000). Anatomical abnormalities including varicoceles, ductal obstructions, or ejaculatory disorders can also contribute to infertility (Olayemi, 2010 as cited in Sinclair, 2000).

On a global scale, male infertility is on the rise (Levigne et al., 2017). Decreasing semen count and quality is observed across the globe with higher impact observed in localized geographic regions. In western populations of New Zealand, Australia, Europe, and North America sperm count and concentration decreased by 59.3% and 52.4% respectively (Levigne et al., 2017). Despite the broad impact on male reproductive health, in about 50% of cases of infertility there is no single distinguishable cause for seminal abnormalities (Kumar et al., 2006). Because many underlying

mechanisms of infertility remain unknown, recent research has focused on the identification of biomarkers and the validation of diagnostic tools for more accurate quantification and diagnosis of male infertility.

It is also notable that male fertility is correlated with overall health outcomes (Braun et al., 2017). It remains unclear if poor overall health lends to reduced semen quality and fecundity, or if decreased fertility is causative of inducing poor general health (Braun et al., 2017); despite this, a growing body of evidence links reduced fertility in men to oncologic, cardiovascular, metabolic, and autoimmune diseases, as well as being predictive of hospitalization rates and all cause mortality (Choy & Eisenberg, 2018; Braunet al., 2017). In one study including over 51,000 infertile couples, male factor infertility results in a threefold higher risk factor of developing testicular cancer (Walsh et al., 2009). Fascinatingly, male infertility has been proven to be an effective biomarker not only for the individual male's health, but for that of his family as well (Choy & Eisenberg, 2018). A 2016 retrospective study including over 26,000 men linked male infertility with increased risk of testicular cancer, thyroid cancer, and acute lymphoblastic leukemia amongst first- and second-degree family members (Choy & Eisenberg, 2018 as cited in Anderson et al., 2016). The strong associations between male factor infertility and overall health call for further investigation into the countless mechanisms involved in the disorder.

Ribosomal Components and Biosynthesis

Ribosomes are the most abundant macromolecules within the cell. 60% of all cellular transcription is devoted to their construction (Salim & Gerton, 2019). Ribosomes

are credited as being the site of protein biosynthesis within the cell and exist in multiple copies within the genome to meet the critical demand for protein synthesis (Salim & Gerton, 2019). These copies are confined in tandem repeats within the genome. *Because of the frequency with which the tandem repeats are transcribed, and the difficulties associated with replication of tandemly repetitive genome sequences, the number of genomic copies encoding the ribosomal components, also regarded as the copy number, are subject to high variability when exposed to genomic stress* (Salim & Gerton, 2019).The number of these tandem repeats varies not only between species, but also between individuals of the same species (Gibbons et al., 2015).

Ribosomes are composed of four rRNAs and 80 ribosomal proteins (Fatica & Tollervey, 2002). The three rRNAs are encoded in ribosomal DNA (rDNA), and form the structural and catalytic components of the ribosome (Fatica & Tollervey, 2002). Three of the rRNAs, 18S, 5.8S, and 28S, originate from a larger precursor rDNA, the 45S array, located on the short arms of chromosomes 13, 14, 15, 21, and 22 (Gibbons et al., 2015). In humans, this 43 kb sequence exhibits a copy number range of 60-800 units in diploid cells. The other rDNA encoding the 5S subunit is located in 2.2 kb tandemly repeated sequences on chromosome 1 and is genetically unlinked from the 45S subunit (Yu and Lemos, 2016; Gibbons et al., 2015). The 5S rDNA array falls within a range of 10-400 units in diploid cells (Yu and Lemos, 2016; Gibbons et al., 2015). Despite the highly variable nature of the 45S and 5S copy numbers and their unlinked positions within the genome, the ribosomal DNA copy numbers (rDNAcn) of the 5S and 45S subunits have been observed to exist in a concerted ratio within both human and mouse models (Gibbons et al., 2015). Once transcribed, the 5S rRNA, 5.8S rRNA, and 28S

rRNA come together to form the larger ribosomal subunit, the 60S. The smaller subunit of the ribosome, the 40S, is made by the 18S rRNA (Gibbons et al., 2015).

The origin of ribosomal construction begins in the nucleolus located within the nucleus of the cell (Fatica & Tollervey, 2002; Thomson et al., 2013). It is here that the transcription of the 18S, 5.8S, and 28S rRNAs is performed by RNA polymerase I (Gibbons et al., 2015; Thomson et al., 2013). These three rRNAs contribute to the core structures of the nucleolus (Gibbons et al., 2015). Transcription of the 5S array by RNA polymerase III is localized to the perimeter of the nucleolus (Gibbons et al., 2015). While not experimentally validated, it is hypothesized that the localization of the 5S transcription to the periphery of the nucleolus is due to evolutionary pressure to maintain the gene balance between the 45S and 5S arrays (Gibbons et al., 2015).

Variation in rDNAcn

The copy numbers of ribosomal subunits are highly variable and susceptible to genomic stress. In fact, rDNAcn varies due to DNA damage and repair, meiotic recombination, environmental exposures, and disease pathologies. The rDNA are the most frequently transcribed loci (Salim & Gerton, 2019). The organization of the genes in tandem repeats, and the frequency with which they are transcribed makes replication transcription conflicts relatively common (Salim & Gerton, 2019). Unequal sister chromatid exchange during mitosis is promoted by stalled replication forks which eventually collapse into double stranded breaks. The repair of these breaks requires recombination dependent repair pathways, and transcription by RNAPI. This process clears cohesion between the tandem repeats, leading to inequalities in inheritance

between sister chromatids (Salim & Gerton, 2019). This process, due to the identical repeats at each locus, make rDNA susceptible to copy number changes mediated by recombination (Salim & Gerton, 2019). Research performed in yeast cells outlines the importance of excess rDNA copies that remain untranscribed to enable the efficient repair of DNA in the rDNA arrays (Carr et al., 2010). A study performed in 2013 identified stress as a causal factor of rDNA array contraction (Hyrien et al.,2013). These fluctuations are critical to the stability of the rDNA, enabling the rescue of transcriptional ability when debilitating mutations are induced by genomic stress (Shyian et al. 2016).

Stress to the nuclear genome, including several environmental factors, have been observed to have significant impact on rDNAcn. In *Daphnia pulex*, exposure to heavy metals altered the copy number of the 18S rDNA subunit over several generations (Harvey et al., 2020). The 18S copy number in lineages not exposed to heavy metals remained stable (Harvey et al., 2020). In the case of heavy metal exposure, DNA damage and repair inhibition is well studied and is hypothesized to be responsible for rDNAcn variation (Harvey et al., 2020, Hengstler et al., 2003). Genomic stress administered by heat shock to *Brassica nigra* was also seen to reduce the gene copies between generations (Waters and Schaal, 1996). The association of heavy metal exposures in variation in rDNAcn has also been observed in humans, where increased metal exposure was associated with changes in the 45S copy number (Feng et al., 2020). While genomic contraction is often observed in instances of genomic stress, expansion of the rDNA arrays has been observed in the cells of cancerous tissue, enabling increased protein production and cell proliferation at an increased rate (Wang and Lemos, 2017; Feng et al., 2020).

Trends also suggest that copy number variation within a population is affected by age (Malinovskaya et al., 2018). In a study from Moscow including 651 individuals, it was determined that while the average rDNAcn remained constant between young and old populations, variation in copy number was reduced significantly in elderly individuals. Additionally, the number of hypermethylated regions in the genomes of older populations was reduced to nearly nothing (Malinovskaya et al., 2018). From these results, researchers put forward two hypotheses. First, individuals with exceptionally high or low rDNAcn do not survive to old ages, leading to a reduction in variation amongst older populations. Secondly, through an unknown mechanism the genome restricts the rDNAcn via the elimination of hypermethylated gene copies (Malinovskaya et al., 2018).

Sperm Transcriptional Activity.

During spermiogenesis, the process of development from round spermatids into elongated mature spermatozoa, protein histones are replaced by protamines (Steger, 1999). Protamines are the predominant nuclear protein in mature sperm. While their function remains largely unknown, it is thought that the packaging of paternal DNA with protamines enables the rearrangement of DNA so that the current genes are expressed in the early embryo. Additionally protamines may serve to protect genes from physical and chemical damage, and aid in faster travel through the female reproductive tract pre fertilization (Braun, 2001). Protamines do not only bind DNA and inhibit transcriptional machinery, but working with histones and transition proteins further compact the chromatin of the sperm further inhibiting transcriptional activity (Bukowska et al., 2013; Steger, 1999).

The tightly wound structure of the DNA protamine complex results in the halt of transcription several days before the end of the spermiogenesis period (Stegar, 1999). Regulated by methylation and trans- acting factors binding to the TATA - box, CREbox and other sequences within the promoter region, transcription is further repressed. While mRNAs are present in mature sperm cells, experiments using radiolabeled uridine triphosphate and cellular analysis RNA supported the notion that mature sperm do not transcribe novel RNAs; instead, RNAs are transcribed pre - maturation and are translationally repressed until later where they may add to the existing RNA pool in the developing oocyte (Grunewald et al., 2005).

Further regulation of transcription in sperm is seen via methylation profiles. The addition of a methyl group to cytosine inhibits proper attachment by transcriptional machinery. Using bisulfite pyrosequencing, the methylation in 18S rDNA, 28S rDNA, and upstream promoter regions was assessed and seen to increase significantly with the age of the individual (Potabattula et al., 2020). This observed pattern is conserved across mammals with vastly different life spans. Researchers hypothesize that methylation in the rDNA of sperm may give insight into the nuclear development of the early embryo (Potabattula et al., 2020).

Sperm Translational Silencing

Despite the long-accepted notion that sperm were translationally inert, recent investigation has shown nuclear genes are expressed as proteins during the sperms movement through the female reproductive tract (Gur & Breitbart, 2006). Using radiolabeled amino acids, incorporation into polypeptides during the capacitation of

sperm was observed; further, inhibition of translation at this stage had adverse impacts on the motility, capacitation, and fertilization rate of sperm cells (Gur & Breitbart, 2006).

Translation in sperm is diminished almost entirely by the cleaving of transcribed rRNA sequences in mature sperm (Johnson et al., 2011). The stark difference in rRNA presence in somatic versus germline cells highlights the frequency with which rRNAs are cleaved shortly after the maturation of the spermatozoa is complete. Additionally, translation is controlled via binding to the poly(A) tails at the 3' ends of mRNAs by poly(A)-binding proteins (PABPs) to regulate and restrict their translational activity (Ozturk & Uysal, 2018).

Related Studies

In recent years, the Environmental Epigenetics laboratory at the University of Massachusetts Amherst has identified mitochondrial DNA copy number (mtDNAcn) a biomarker for male fertility and has verified increased mtDNAcn as a predictor of male infertility (Rosati et al., 2020). Other studies have identified variation in both mtDNAcn and rDNAcn in the context of diseased tissues such as gastric tumors (Feng et al., 2020). Additionally, chronic exposure to heavy metals has been associated with a reduction in rDNAcn in *Daphnia pulex* (Harvey et al., 2020). Taken together, these findings suggest that rDNAcn may serve as a "canary in a coalmine" predictor of reduced genetic fitness. Our research investigated associations between semen parameters and the relative copy numbers of the 18S and 5S arrays. Additionally, we sought to investigate the possible association between ribosomal rDNAcn, and mtDNAcn and mtDNAdel.

Materials and Methods:

Study Population and Semen Collection. The population for this study consisted of males between the ages of 18-55 years old. These individuals were recruited as members of the Sperm Environmental Epigenetics and Development Study (SEEDS) between 2014-2016, at the Baystate Reproductive Medicine Clinic in Springfield, MA (Wu et al. 20107). Whole semen samples (n=148) were collected, and data was collected by clinical personnel at the Baystate Medical Center including data on semen parameters, demographics, lifestyle factors, and medical history for participating individuals.

Semen DNA Isolation. Semen samples were collected in a sterile collection cup after a 2-3 day abstinence period, and transferred to the university of Massachusetts. Here they were stored at -80°C prior to processing. To separate motile sperm, whole semen samples were processed using a two-step gradient fractionation. From the motile sperm, DNA was isolated through a previously published protocol (Wu, 2015).

Sperm rDNA Quantification. A triplex qPCR protocol was developed to enable the assessment of the 5S and 18S rDNA arrays in concurrence with the p53 single copy gene. Table 1 shows the forward and reverse primers and probe sequences for the 5S, 18S, and P53 DNA arrays. P53 was chosen as a reference for single copy genes in the nuclear genome because of its low variability, which enables its use in determining the copy numbers of other genes. Standard curves were run using a 1:2 serial dilution with 5 more standards prepared from the highest concentrated standard.

Super Master mix was made using the following:

- 5 uL Taqman Master Mix (IDT # 00997739)
- 0.5 uL 5S rDNA Primer Mix at (5uM)
- 0.5 uL 18S rDNA Primer Mix at (1uM)
- 0.5 uL p53 Primer Mix at (1uM)
- 1.5 uL Nuclease Free Water (IDT, Coralville, IA, USA)

Samples were run in triplicate in a 96 well plate. Each well contained 8 uL of the super master mix as well as 2 uL DNA. For blank controls, 2 uL of nuclease free water was used in place of the DNA. The following cycling conditions were followed:

Holding Stage:

90°C, 10 minutes

Cycling Stage:

95°C, 15 seconds

60°C, 45 seconds

PCR efficiency was calculated as:

$$\text{PCR efficiency} = 10^{(-1/\text{slope})}-1$$

Sample DNA concentration was read using a fluorometer and diluted to 5ng/uL before use in triplex qPCR analysis. Each sample was run in triplicate, and the copy number was determined by:

$$\text{rDNA target copy number} = 2^{(\text{p53 CT} - \text{rDNA target CT})}$$

CT scores were determined for each reaction and averaged to determine CT difference values. A water control, fertile standard, and infertile standard were run on each plate. Each reaction contained 8 uL of Super Master Mix and 2uL of diluted DNA.

Data Analysis

Data collected through the triplex qPCR reactions were compiled with data from the SEEDS Study. The Baystate Medical Center provided evaluations on semen parameters as well as potential covariates. Data analysis was performed through R data analysis software. The 18S and 5S rDNAcn were skewed right, so logarithmic conversion was used to normalize the data. Bivariate spearman correlations were performed to compare 18S rDNAcn and 5S rDNAcn with semen parameters including concentration, count, motility, and morphology. Gaussian correlation was performed to determine potential confounders in age, BMI, and race, and corrected models were run to assess significance after adjustments. Analysis of Variance (ANOVA) and Post Hoc analysis were used to determine the significance of copy numbers in regards to increased fertility. Ribosomal copy number was also assessed against mitochondrial copy number and deletions using spearman bivariate correlations.

Results and Discussion

Both 18S and 5S rDNAcn were rightward skewed, with mean copy numbers of 31.46±20.6, and 8.01±1.75 respectively. Logarithmic conversion was used to normalize the data before analysis. Spearman bivariate analysis indicated that 5S copy number

was weakly correlated with concentration (rho = -0.221, p = 0.0096) and motility (rho = -0.22, p = 0.0062) with borderline significance. Count and morphology do not appear to be associated with 5S copy number (Figure 1) Spearman bivariate analysis of the 18S rDNAcn showed a moderately strong association between concentration (rho = -0.447, p = 4.52e-08), count (rho = -0.44, p = 7.34e-08), morphology (rho = -0.34, p = 5.13 e-05), and motility (rho =- 0.389, 2.7e-06) (Figure 2).

Table 2 shows the relationship between log transformed 5S copy number, and semen parameters obtained through gaussian analysis. Of the four semen parameters assessed, only motility showed significance with a p - value of 0.005 (significance cutoff of $p < 0.05$). No associations were found between ribosomal copy number and age, BMI, or race. Corrected models were run to further ensure that confounding variables were corrected. When adjusted for age, BMI, and race, motility remained significant, although the p - value increased to 0.044 (Table 3). Table 4 shows the relationship between log transformed 18S copy number, and semen parameters obtained through gaussian analysis. All four semen parameters, concentration, count, motility, and morphology were significantly associated with log(18Scn). Correction of the model for age, BMI, and age resulted in increased p values (Table 5). These p values remained well below to significance cutoff ($p < 0.05$). The confidence intervals encompassed significant results as they did not include zero within the suggested CI ranges.

Further analysis was performed through the construction of a ranking system (table 6) which ranked individuals by the number of semen parameters for which they were sufficiently high to be above the WHO cut off for fertility. Boxplots were constructed to visualize the distribution of copy numbers between groups of increasing

fertility. Figure 4 indicates that there is no significant difference between fertility groups in regard to the 5S rDNAcn. ANOVA, ptrend, and post hoc analysis do not indicate statistically significant variation ($F = 2.472$, $p = 0.089$, $ptrend = 0.077$).

Analysis of the 18S rDNAcn by post hoc analysis showed significant variation between groups R1 and R2 ($p = 2.6 e-05$) as well as between R1 and R3 ($p =4.3e-07$) (Figure 3). Additionally, ANOVA and p trend analysis showed significant variation between groups ($F = 15.53$, $p = 9.33e-07$, p trend = 1.31 e-05). The significant p trend value suggests that there is a linear trend between the copy number and the quality of a semen sample.

Comparison of the ribosomal 5S and 18S rDNAcn values vs mitochondrial DNA copy number (mtDNAcn) and deletions (mtDNAdel) were also performed using bivariate spearman analysis. The 5S rDNAcn showed no significant relationship between mtDNAcn ($rh = 0.043$, $p = 0.636$) or mtDNAdel ($rho = 0.118$, $p = 0.191$) (Figure 5). The 18S rDNAcn was moderately correlated with mtDNAcn ($rho = 0.4$, $p = 9.12e-07$) and mtDNAdel (rho = 0.41, 1.78e-06) (Figure 5).

A moderately strong inverse relationship was found between the 18S rDNAcn and semen parameters. This relationship suggests that rDNAcn may have some predictive power for fertility. The relationship between rDNAcn and mtDNAcn coupled with the strength of mtDNAcn as a predictor of fertility may also suggest some mechanism of crosstalk between mitochondria and ribosomes. The mechanism of this relationship is still unknown but gives insight into the complex nature of the disorder and the impact of copy number variation.

Conclusions and Future Directions

Analysis of the 18S and 5S ribosomal subunits in 140 individuals indicates that ribosomal gene dosage, particularly of the 18S subunit, is inversely correlated with semen parameters. In addition, we found that 18S rDNAcn is positively correlated with mtDNAcn and mtDNAdel, two known predictors for infertility. Our findings give insight into the complex mechanisms which contribute to male factor infertility and suggest some manner of crosstalk between ribosomal and mitochondrial DNA.

The underlying mechanism by which the 18S rDNAcn and semen parameters are related is unclear; however, there are several possible biological reasons that this relationship may exist. More copies of ribosomal genes may be indicative of heightened levels of cellular damage and overactive DNA repair mechanisms (Kobayashi et al., 2014). Due to unequal sister chromatid exchange, DNA repair mechanisms often result in genes being inserted more than once. Through this mechanism, cells with high levels of cellular trauma or genetic stress may have highly variable ribosomal copy numbers (Kobayashi et al., 2014). Additionally, we may hypothesize that the requirement for functional sperm cells to be transcriptionally and translationally down regulated is impeded by high levels of ribosomal components (Johnson et al., 2011).

Further analysis is needed amongst general population samples to further corroborate rDNAcn as a single measure indicator of sperm quality. Additionally, future exploration into associations between ribosomal genes and ART outcomes to assess the impact of ribosomal gene dosage on embryo quality and live birth. Because of the

known increased methylation with age in the upstream promoter region, exploration into methylation profiles and their relationship with semen parameters and environmental exposures such as BPA and phthalates is a potential area of future research as well (Potabattula et al., 2020).

Tables and Figures

Table 1. Primer and probe sequences used in quantitative polymerase chain reaction (qPCR)

Prmier Sequences:		
5S rDNA Array		
	Forward	5'- GCC CGA TCT CGT CTG ATC-3'
	Reverse	5'- GGT TT CCC AGG TC-3'
18S rDNA Aray		
	Forward	5'-CACCCACGGAATCGAGAAA-3'
	Reverse	5'-TCAACACGGGAAACCTCAC-3'
p53 DNA Array		
	Forward	5'-CTTCTCACTTCCACGACTGAC-3'
	Reverse	5'-GAGCGATCTTTCCAGGCA-3'
Probe Sequences:		
5S:		5'-/5ATTO550N/ATC CCA GTA CTA ACC AGG CCC GAC/3IAbRQSp/-3'
18S:		5'-/5SUN/CGGACACGG/ZEN/ACAGGATTGACAGATT/3IABkFQ/-3'
p53:		5'-/56-FAM/CCGGCTCCG/ZEN/CTAGATGGAGAAA/3IABkFQ/-3'

Table 2. Linear regression of log5S copy number vs semen parameters in unadjusted model.

	Coefficient	p - value	CI
Morphology	-2.095	0.64	-10.89, 607
Motility	-50.72	0.005 *	-86.12, -15.31
Concentration	-137.6	0.112	-307.6, 32.4
Count	-137.3	0.462	-504.8, 230.32

Table 3. Linear regression of log5S copy number vs semen parameters corrected for age, BMI, and race.

	Coefficient	p - value	CI
Morphology	-0.285	0.966	-1.57, 13.00
Motility	-55.988	0.044 *	-110.44, -1.53
Concentration	-132.204	0.239	-354.52, 90.11
Count	-191.478	0.546	-822.20, 439.25

Table 4. Linear regression of log18S copy number vs semen parameters in unadjusted model.

	Coefficient	p - value	CI
Morphology	-6.7	6.72 e -06 *	-9.9, -4.03
Motility	-31.69	6.71 e -07 *	-11.2, -3.2
Concentration	-714.9	1.76 e -04 *	-173.7, -55.99
Count	-299.76	3.77 e -06 *	-422.7, - 176.86

Table 5. Linear regression of log18S copy number vs semen parameters corrected for age, BMI, and race.

	Coefficient	p - value	CI
Morphology	-375.51	6.28e-04 *	-11.20, -3.21
Motility	-31.32	3.69e-04 *	-47.97, -14.67
Concentration	-126.86	3.04e-04 *	-193.23, -60.49
Count	-375.52	1.33e-04 *	-560.1, -190.94

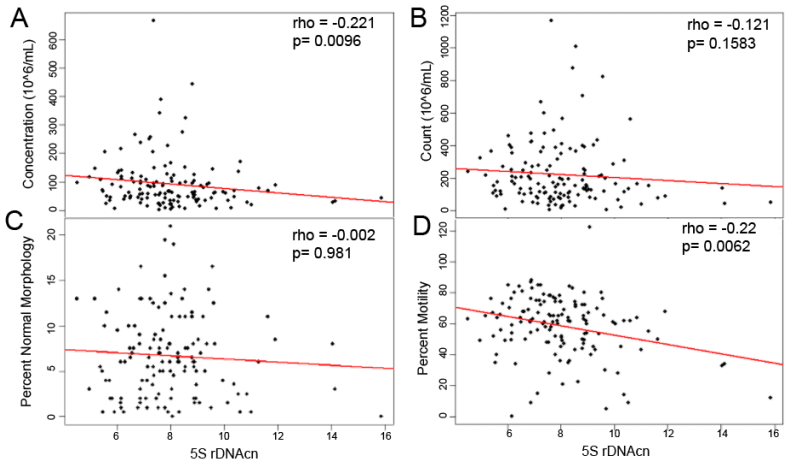

Figure 1. Linear regression of 5S rDNAcn vs concentration (A), count (B), morphology (C) and motility (D). Rho values suggest a weak negative relationship. Statistical significance is present within the concentration and motility models ($p < 0.05$). Count and morphology are not statistically significant.

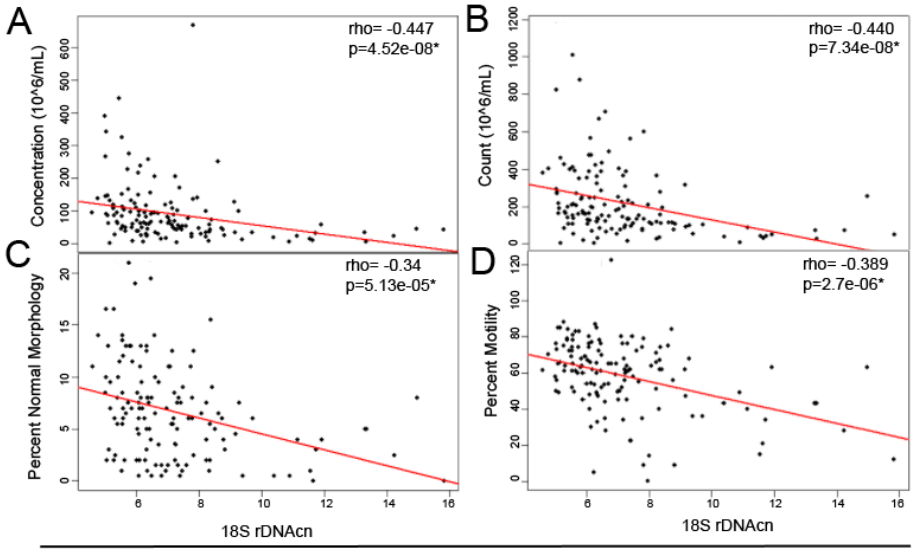

Figure 2. Linear regression of 18S rDNAcn vs concentration (A), count (B), morphology (C) and motility (D). Rho values suggest a moderatly strong negative relationship. Statistical significance is present within all four models ($p < 0.05$).

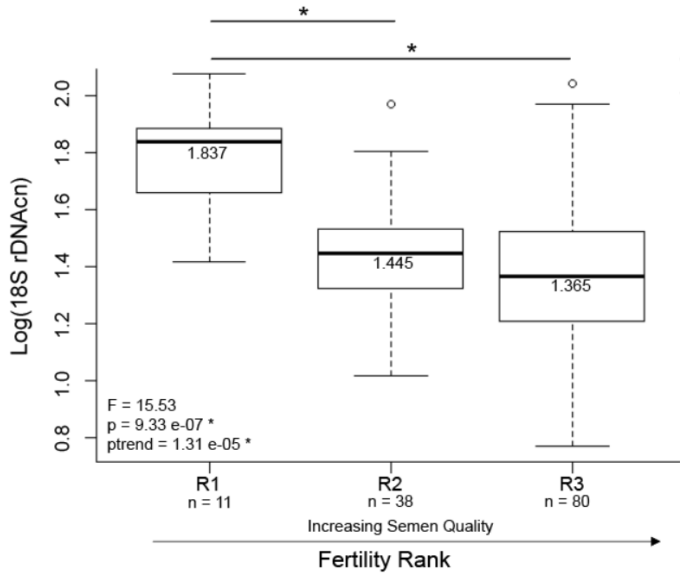

Figure 3. Log(18Scn) vs ranked fertility. Posthoc analysis showed significant difference between group R1 and R2 as well as between R1 and R3 ($p < 0.05$). ANOVA and ptrend analysis also showed statistical significance suggesting a linear relationship between log(18Scn) and fertility ranking.

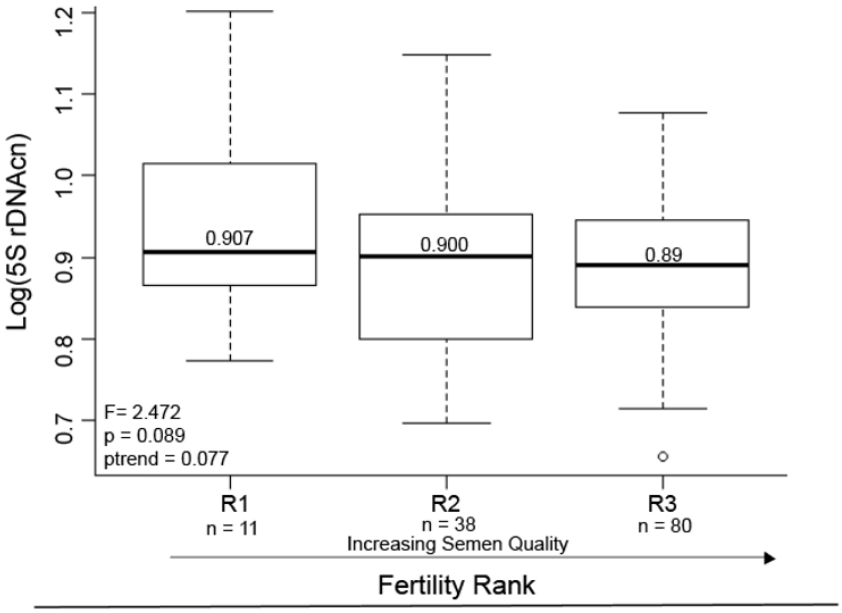

Figure 4. Log(5Scn) vs ranked fertility. Posthoc analysis showed no significant difference between groups. ANOVA and ptrend analysis also showed no statistical significance suggesting the absence of a linear relationship between log(18Scn) and fertility ranking.

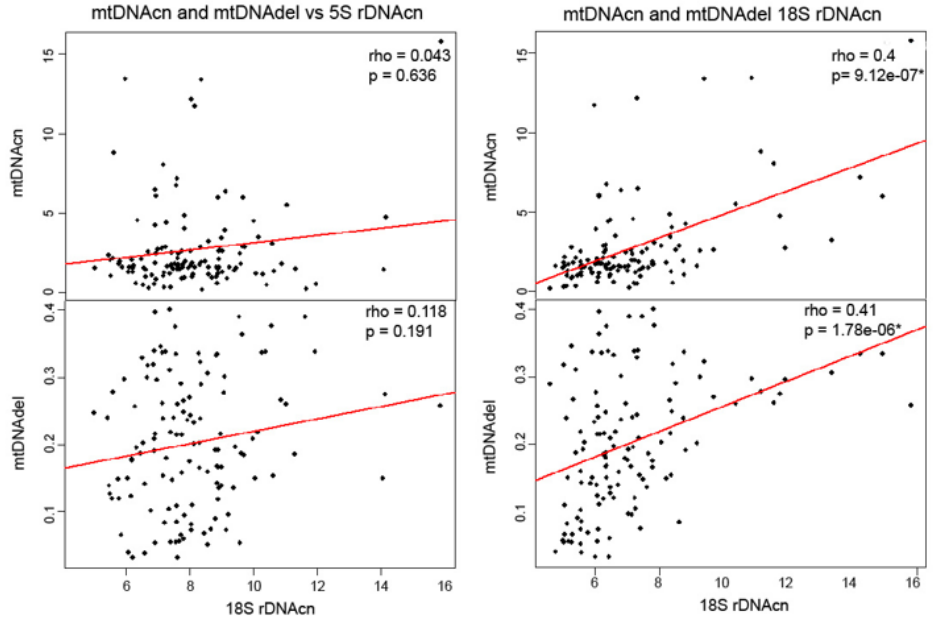

Figure 5. 5S and 18S rDNAcn vs mtDNAcn and mtDNAdel. No significant relationship is seen within the 5S correlation plot. There is a positive and significant association between the 18S rDNAcn and both mtDNAcn and mtDNAdel.

References

Bonde, J., Eisenberg, M., Giwercman, A., Hærvig, K., Rimborg, S., Vassard, D., Pinborg, A., Schmidt, L., Bräuner, E., & Glazer, C. (2017). Male Infertility and Risk of Nonmalignant Chronic Diseases: A Systematic Review of the Epidemiological Evidence. *Seminars in Reproductive Medicine*, *35*(03), 282–290. https://doi.org/10.1055/s-0037-1603568

Braun, R. E. (2001). Packaging paternal chromosomes with protamine. *Nature Genetics*, *28*(1), 10–12. https://doi.org/10.1038/ng0501-10

Bukowska D, Kempisty B, Piotrowska H, Sosinska P, Wozna M, Cieliolka S, Antosik P, Jaskowski JM, Brussow KP, Nowicki M. The structure and role of mammalian sperm RNA: a review. Veterinari Medicina. 2013 Feb; 85(2): 57-64.

Carr, A. (2007). Faculty Opinions recommendation of Abnormality in initiation program of DNA replication is monitored by the highly repetitive rRNA gene array on chromosome XII in budding yeast. *Faculty Opinions – Post-Publication Peer Review of the Biomedical Literature*. https://doi.org/10.3410/f.1050629.520642

Choy, J. T., & Eisenberg, M. L. (2018). Male infertility as a window to health. *Fertility and Sterility*, *110*(5), 810–814. https://doi.org/10.1016/j.fertnstert.2018.08.015

Fatica, A., & Tollervey, D. (2002). Making ribosomes. *Current Opinion in Cell Biology*, *14*(3), 313–318. https://doi.org/10.1016/s0955-0674(02)00336-8

Feng L, Du J, Yao, C, Jiang Z, Li T, Zhang Q, Guo X, Yu M, Xia H, Shi L, Jia J, Tong Y, Ju L, Liu J, Lou J. Ribosomal DNA copy number is associated with P53 status and levels of heavy metals in gastrectomy specimens from gastric cancer patients. Environ Int. 2020 May; 138:

Johnson GD, Sendler E, Lalancette C, Hauser R, Diamond MP, Krawetz SA. Cleavage of rRNA ensures translational cessation in sperm at fertilization. Mol Hum Reprod. 2011 Dec; 17(12): 721-726.

Jungwirth A, Diemer T, Kopa Z, Krausz C, Minhas S, Tournaye H. EAU Guidelines on Male Infertility. European Association of Urology. 2018: 5-7.

Harvey EF, Cristescu ME, Dale J, Hunter H, Randall C, Crease TJ. Metal exposure causes rDNA copy number to fluctuate in mutation accumulation lines of Daphnia pulex. Squat Toxicol. 2020 Sep; 226: 105556.

Hengstler, J. G. (2003). Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove co-exposures to cadmium, cobalt and lead as more dangerous than hitherto expected. *Carcinogenesis*, *24*(1), 63–73. https://doi.org/10.1093/carcin/24.1.63

Gibbons, JG, Branco, AT, Godinho, SA, Yu S, & Lemos B. Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. Proceedings of the National Academy of Sciences. 2015; 112(8), 2485-2490. doi:10.1073/pnas.1416878112

Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. Genes dev. 2006 Feb 15; 20(4): 411-416 .doi: 10.1101/gad.367606

Hyrien, O. (2013). Faculty Opinions recommendation of A natural polymorphism in rDNA replication origins links origin activation with calorie restriction and lifespan. *Faculty Opinions – Post-Publication Peer Review of the Biomedical Literature*. https://doi.org/10.3410/f.718011072.793476833

Kumar, R., Gautam, G., & Gupta, N. P. (2006). Drug Therapy for Idiopathic Male Infertility: Rationale Versus Evidence. *Journal of Urology*, *176*(4), 1307–1312. https://doi.org/10.1016/j.juro.2006.06.006

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: a systematic review and meta-regression analysis. *Human Reproduction Update*, *23*(6), 646–659. https://doi.org/10.1093/humupd/dmx022

Malinovskaya EM, Ershova ES, Golimbet VE, Porokhovnik LN, Lyanpunova NA, Kutsev SI, Veiko NN, Kostyuk SV. Copy Number of Human Ribosomal Genes With Aging: Unchanged Mean, but Narrowed Range and Decreased Variance in Elderly Group. Front Genet. 2018; 9: 306.

Olayemi, F. O. (2010, May 17). Review of *A review on some causes of male infertility*. *African Journals Online*, *9*(20).

Potabattula R, Zacchini F, Ptak GE, Dittrich M, Muller T, Hajj NE, Hahn T, Drummer C, Behr R, Lucas-Hahn A, Niemann H, Schorsch M, Haaf T. Increasing methylation of sperm rDNA and other repetitive elements in the aging male mammalian germline. Aging Cell. 2020 Aug; 19(8); e13181.

Rosati AJ, Whitcomb BW, Brandon N, Buck Louis GM, Mumford SL, Schisterman EF, Pilsner JR. Sperm mitochondrial DNA biomarkers and couple fecundity. Human Reproduction 2020. 35(11): 2619-2625.

Salim, D., & Gerton, J. L. (2019). Ribosomal DNA instability and genome adaptability. *Chromosome Research*, *27*(1-2), 73–87. https://doi.org/10.1007/s10577-018-9599-7

Thomson E, Ferreira-Cerca S, Hurt E. Eukaryotic ribosome biogenesis at a glance. Journal of Cell Science. 2013; 126: 4815-4821

Shyian, M., Mattarocci, S., Albert, B., Hafner, L., Lezaja, A., Costanzo, M., Boone, C., & Shore, D. (2016). Budding Yeast Rif1 Controls Genome Integrity by Inhibiting rDNA Replication. *PLOS Genetics*, *12*(11). https://doi.org/10.1371/journal.pgen.1006414

Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol. 1999 Jun; 199(6): 471-487.

Waters ER, Schaal BA. Heat shock induces a loss of rRNA-encoding DNA repeats in Brassica nigra. Proc Natl Acad Sci USA. 1996 Feb; 93(5=4): 1449-1452.

Walsh, T. J., Croughan, M. S., Schembri, M., Chan, J. M., & Turek, P. J. (2009). Increased Risk of Testicular Germ Cell Cancer Among Infertile Men. *Archives of Internal Medicine*, *169*(4), 351. https://doi.org/10.1001/archinternmed.2008.562

Wu H, Whitcomb BW, Huffman A, Brandon N, Labrie S, Tougias E, Lynch K, Rahil T, Sites CK, Pilsner JR. Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting. Hum Reprod. 2019 Jan 1; 34(1):163-170. doi: 10.1093/humrep/dey330. PMID: 30428043; PMCID: PMC6295960.

Yu S, Lemos B. A Portrait of Ribosomal DNA Contacts with Hi-C Reveals 5S and 45S rDNA Anchoring Points in the Folded Human Genome. Genome Biol Evol. 2016 Nov; 8(11): 3545-3558. doi: 10.1093/gbe/evw257