Epidemiologic and Molecular Relationship Between Vaccine Manufacture and Autism Spectrum Disorder Prevalence

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ABSTRACT

Objectives: **To assess the public health consequences of fetal cell line manufactured vaccines that contain residual human fetal DNA fragments utilizing laboratory and ecological approaches including statistics, molecular biology and genomics.**

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Transparency declaration: The lead author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis

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Method: MMR coverage and autism disorder or autism spectrum disorder prevalence data for Norway, Sweden and the UK were obtained from public and government websites as well as peer reviewed published articles. Biologically, the size and quantity of the contaminating fetal DNA in Meruvax®II and Havrix® as well as the propensity of various cell lines for cellular and nuclear uptake of primitive human DNA fragments were measured and quantified using gel electrophoresis, fluorescence microscopy and fluorometry. Lastly, genomic analysis identified the specific sites where fetal DNA fragment integration into a child's genome is most likely to occur.

Results: The average MMR coverage for the three countries fell below 90% after Dr. Wakefield's infamous 1998 publication but started to recover slowly after 2001 until reaching over 90% coverage again by 2004. During the same time period, the average autism spectrum disorder prevalence in the United Kingdom, Norway and Sweden dropped substantially after birth year 1998 and gradually increased again after birth year 2000. Average single stranded DNA and double stranded DNA in Meruvax®II were 142.05 ng/vial and 35.00 ng/vial, respectively, and 276.00 ng/vial and 35.74 ng/vial in Havrix® respectively. The size of the fetal DNA fragments in Meruvax®II was approximately 215 base pairs. There was spontaneous cellular and nuclear DNA uptake in HFF1 and NCCIT cells. Genes that have been linked to autism (autism associated genes; AAGs) have a more concentrated susceptibility for insults to genomic stability in comparison to the group of all genes contained within the human genome. Of the X chromosome AAGs, 15 of 19 have double strand break motifs less than 100 kilobases away from the center of a meiotic recombination hotspot located within an exon.

Conclusion: Vaccines manufactured in human fetal cell lines contain unacceptably high levels of fetal DNA fragment contaminants. The human genome naturally contains regions that are susceptible to double strand break formation and DNA insertional mutagenesis. The "Wakefield Scare" created a natural experiment that may demonstrate a causal relationship between fetal cell-line manufactured vaccines and ASD prevalence.

Keywords: Autism, vaccine, meiotic recombination hotspot, DNA uptake, fetal cell lines

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A worldwide autism epidemic is copiously established by the number of peer reviewed articles on the subject, including the observations from numerous institutions that de novo genetic insertions and mutations are excessive in children with autism.[1] Autism disorder (AD), a subset of Autism Spectrum Disorder (ASD), is a neurological

and developmental disorder whose symptoms usually appear within the first three years of life.[2] The autism epidemic obviously creates significant public health burden and demands critical assessment of environmental factors that may trigger this epidemic. A previous publication from our group focused on overlooked, universally introduced environmental factors, including human fetal and retroviral contaminants in childhood vaccines, advancing paternal age and changes in diagnostic criteria. As the US Environmental Protection Agency (EPA) requires, discovery of potential environmental triggers for autism requires statistical assessment to identify birth year change points for autism spectrum disorder prevalence. Iterative fitting algorithms identified 1980.8, 1988.4 and 1996.5 as "changepoint" years for the United States AD prevalence,[3] in substantiation of a report from the Environmental Protection Agency (EPA) that identified a 1988 worldwide AD change point.[4] Additionally, AD birth year changepoints for the United Kingdom, Western Australia and Denmark are calculated to be 1987, 1990.4 and 1987.5, respectively.[3] These statistically calculated AD changepoints do not correspond to changepoints that would be predicted based on printing schedules for revisions to the Diagnostic and Statistical Manual (DSM), and therefore DSM revisions cannot be the primary environmental or sociological trigger responsible for current AD prevalence. Advancing paternal age is currently a favored explanation for the worldwide autism epidemic. However, linear regression analysis for the US and Western Australia data revealed no relationship between paternal age and AD for any specific birth year.[3]

In UK and some Scandinavian countries it is notable that there was a significant decline in MMR immunization coverage during discrete years hypothetically triggered by a scare following Andrew Wakefield's 1998 publication[5] that suggested MMR immunization and autistic regression were linked, but clearly not due to a failure to report immunizations.[6-8] Since we detected a relationship between MMR and AD/ASD prevalence in our previous study, the observation that MMR coverage dropped acutely in UK and Scandinavian countries during discrete years raises the question of whether MMR coverage impacted AD/ASD prevalence in these countries during this same time period .

Utilizing human fetal cell lines to manufacture childhood vaccines leaves behind residual human DNA as well as human endogenous retrovirus K (HERVK) fragments in the final vaccine products we inject into our children. Mammalian cells take up same species extracellular DNA fragments via receptor mediated endocytosis. Uptake is most efficient at low concentrations of extracellular DNA[9] and peaks 2 hours after addition of the DNA fragments to cell culture.[10] In the extracellular concentration range of 0.1 to 7 μM, oligonucleotides (small bits of nucleic acids) readily enter cultured cells through receptor mediated uptake,[11-13] reaching intracellular and nuclear[11,14-16] concentrations which equal or exceed that of the extracellular medium within 2-4 hours.[17] Empirical experiments have shown that addition of placental DNA fragments of 500 base pairs in length contributed approximately 4% of a cell's genomic content per hour of incubation roughly 40-50% of fragmented DNA added to cell culture will be taken up by a cell and 10-20% of the added DNA will be delivered to the nucleus, demonstrating the rapidity

with which DNA can enter a cell.[9,18] Current FDA guidance is that the level of residual cell-substrate DNA should be less than 10 ng per vaccine dose with a median DNA size of 200 base pairs or lower.[19]

Genetic analysis of ASD on an individual basis has identified hundreds of diverse de novo mutations, deletions, and duplications present in 48.4% to 63% of cases of autism in which only one child in a family was affected, called simplex ASD.[20] The genomic data analyzed in most publications on this topic is from the Simon's Simplex Collection (SSC), in which 73% of the exomes are covered. Therefore, extrapolating from the reported percentage of children with deleterious mutations, 66% to 86% of simplex ASD would be expected to have a de novo mutation if the entire exome was sequenced. Since 85% of disease causing mutations occur in the exome and 15% are found in regulatory regions, we can therefore estimate that were the regulatory regions also sequenced, 76% to 99% of simplex ASD children would have de novo deleterious mutations.[21] Unaffected children and multiplex ASD, affecting multiple children in a family, have dramatically fewer de novo mutations, however, the majority of children with ASD are simplex (75% to 90%),[22,23] demonstrating the critical role of de novo gene mutations in the worldwide autism epidemic. In other words, susceptibility for autism in simplex cases originates from a de novo source, indicating environmental influences as a major component of ASD, rather than heredity.[24] In fact, recent publications have shown that many human diseases in addition to ASD are associated with de novo chromosomal translocations, megabase-sized deletions, DNA insertions, and duplications.[24] Although ASD is highly heterogeneous in terms of genes and mutations, the affected genes have been demonstrated to interact within a few critical biologic networks, explaining the common phenotype of ASD despite diverse mutations.[20,25] An even more crucial question to be asked is; "What causes these hundreds of diverse de novo mutations to arise?"

During meiosis genomic material is exchanged between the maternal and the paternal chromosomes, a process called meiotic recombination (MR). Hotspots are sites in the genome of varying length where MR occurs most frequently. This process creates genetic diversity in our offspring, and is beneficial in that sense. The human genome contains over 25,000 known recombination hotspots.[26] Curiously, regions of the genome where meiotic recombination has occurred (hotspots) have been shown to be highly predisposed to subsequent somatic cell double strand breaks (DSB) and disease causing mutations,[27-29] including single nucleotide variation, copy number variation, gene deletion events, and insertion/integration of foreign DNA during DSB repair.[30,31] Diseases known to be influenced by genomic deletions or insertions include cancer, hereditary neuropathy,[32] mitochondrial syndromes,[26] ichthyosis,[26,33] Nijmegen breakage syndrome (NBS),[34] autism spectrum disorder,[24,35] schizophrenia[36] and others. In this study we have asked whether MR hotspots which predispose to subsequent somatic mutations are related to the gene mutations that have been linked to ASD.

This study assesses the public health consequences of vaccines contaminated with residual human fetal DNA fragments utilizing laboratory and ecological approaches

including statistics, molecular biology and genomics. Using data available from public and government websites, we have tracked MMR coverage and AD/ASD prevalence in Norway, Sweden and the UK in order to determine whether the decreased MMR®II compliance reported during 1999-2002, after the 'Wakefield Scare', impacted AD/ASD incidence for children born during those years. We also report here the size and quantity of the contaminating fetal DNA in Meruvax®II and Havrix® as well as the propensity of various cell lines for cellular and nuclear uptake of primitive human DNA fragments similar in size and quality to the Meruvax®II fetal DNA fragments. Lastly, our genomic analysis identified the specific sites where fetal DNA fragment integration into a child's genome is most likely to occur. This study is one of the first laboratory and ecological studies conducted that has examined the relationship between human fetal cell line manufactured vaccines, cellular DNA damage, and the world wide autism epidemic.

Methodology

Data sources for Autism Spectrum Disorder Rates and MMR coverage

National MMR coverage in Norway was obtained from the Norwegian Institute of Public Health,[37] for Sweden from the National Board of Health and Welfare[38] and for UK from the Public Health England,[39] MMR vaccine coverage was plotted as percent of maximum coverage in each country.

Autism spectrum disorder rates for Norway were assembled using data from three papers: the first was a 2012 publication by Isaksen et al;[40] the second and third were 2012 and 2013 articles both by Suren et al.[41,42] Norwegian population data was obtained from the official website of Statistiska Centralbyran - Statistics Norway.[43] In Sweden, AD rate was obtained from a study published in 2012 "The Prevalence of Autism Spectrum Disorders in Toddlers: A Population Study of 2-Year-Old Swedish Children" by Nygren et al.[44] Swedish population data was obtained from the official website of Statistiska Centralbyran - Statistics Sweden.[45] In the UK, AD rate was assembled from two studies: the first published by Lingam et al. (2003)[46] and the second by Latif et al. (2007).[47] UK population data was obtained from the website of the Office of National Statistics.[48]

Residual DNA quantification in Meruvax®II (Rubella vaccine) and Havrix® (Hepatitis A vaccine)

Rubella Virus Vaccine Live Meruvax®II (Merck & Co. Inc) was dissolved in 30 µl 1X Tris EDTA (TE, pH 8) and incubated at 60°C for two hours to inactivate live virus. Havrix® (GlaxoSmithKline Biologicals) in solution was also heat inactivated at 60°C for two hours. Notably, the single–stranded RNA (ssRNA) virus in Meruvax®II is encapsulated and the ssRNA virus in Havrix® is encapsulated after host entry,[49] therefore it is extremely unlikely that there will be significant levels of free single stranded viral RNA in the vaccines. In fact, to detect rubella virus RNA from oral samples known to be rubella positive, the RNA must be extracted from the encapsulated virus using Qiagen's viral RNA extraction kits,[50,51] which requires capsule lysis under highly denaturing conditions. Furthermore, even if some free viral RNA was present, the DNA

quantitation assay PicoGreen® specificity for dsDNA is 9 fold greater than ssDNA and 31 fold greater than for RNA.

M13 Primer (Life Technologies) was used to make single stranded DNA (ssDNA) standard solutions and Human DNA (Paragon Dx, USA) was used to make double stranded DNA (dsDNA) standard solutions for fluorimetric analysis. Quant-iT™ OliGreen® (Life Technologies) was used to label ssDNA, and Quant-iT™ PicoGreen® (Life Technologies) was used to label dsDNA. The limit of detection of PicoGreen® for dsDNA is 100 pg/ml. OliGreen® serial dilutions of standard DNA solution were made by mixing with Quant-iT™ OliGreen® or Quant-iT™ PicoGreen® for final concentrations of 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml. 200 µl of each serial dilution solution was pipetted into a 96 well plate. Vaccines were diluted by 1, 2, 10, and 100 fold in mixtures of 1X TE with Quant-iT™ OliGreen® or Quant-iT™ PicoGreen®, and 200 µl pipetted into 96 well plates. Fluorescence intensity was analyzed using Gemini EM Microplate Reader (Molecular Device). The DNA contents in each vaccine were calculated based on the standard curves.

Characterization of residual DNA fragment size in Meruvax®II and Havrix® by gel electrophoresis

5 ng of residual human DNA from each vaccine, as quantified previously, was mixed with loading dye (Lonza) and 1X TE in total volume less than 40 µl. Residual human fetal DNA samples and DNA ladder solution (20 base pairs Extended Range DNA Ladder (Lonza) in loading dye and 1X TE for a final concentration 200 ng/µl in total volume 15 µl) were loaded in 4% agarose gels and run over the gel at 210 V for 4 hours. After electrophoresis, the gel was incubated with SYBR Gold staining solution diluted into 1:10,000 with 1X TE overnight at 4°C. DNA bands were imaged under UV light exposure.

The residual fetal DNA in Havrix® didn't migrate from the well, even after one hour 50°C treatment with proteinase K, demonstrating that the fetal DNA fragments in Havrix® are at least greater than 1000 base pairs in length. Further comparison of Havrix® fetal DNA fragments with lambda phage DNA indicates that the fetal DNA fragments are even greater than 48,502 base pairs in length.

DNA uptake and incorporation in host genome in various cell lines.

Human Cot1 DNA (Invitrogen) was labeled using Mirus Label IT CyTM3 Labeling Kit (Mirus), and kept at -20°C until it was used. Cot1 DNA was utilized to detect cellular and nuclear DNA fragment uptake because the fragment size of the Invitrogen human Cot1 DNA product is similar to the fetal DNA fragment size found in Meruvax®II, and because as a cancer cell line it has reverted epigenetically towards undifferentiated primitive fetal type cells.[52]

U937 cells (Histocytic Lymphoma) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37°C under a humidified atmosphere containing 5% CO_2/95% air.

HL-60 cells (myeloblast) were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% antibiotic-antimycotic solution at 37°C under the same condition. Loosely adherent NCCIT (teratocarcinoma) cells were grown in RPMI-1640 supplemented with 10% FBS and 1% antibiotic-antimycotic solution with a cell density 3×10^4 per well of a 24-well plate which a tissue culture treated glass cover slip was placed in each well at 37°C under a humidified atmosphere containing 5% CO_2/95% air. Human Foreskin Fibroblast 1 (HFF1) cells were grown in DMEM supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution was used as a medium. BE (2)-C (neuroblastoma) cells were grown in a 1:1 mixture of Eagle's Minimum Essential Medium (EMEM) and F12 Medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution. M059K (Glioblastoma-Double Stranded Break repair proficient) and M059J (Glioblastoma-Double Stranded Break repair deficient) were also grown in a 1:1 mixture of DMEM and Ham's F12 Medium supplemented with 10% FBS, 0.05 mM non-essential amino acids, and 1% antibiotic-antimycotic solution.

After cells were cultured in each condition for 2 to 3 days 500-750 ng Cy3 labeled human Cot1 DNA was added per 1.0×10^7 cells and incubated at 37°C under a humidified atmosphere containing 5% CO_2/95% air by gently shaking for 24 to 48 hours. The nuclei were then stained with Hoechst stain, German glass cover slips were placed on glass slides, and cellular and nuclear DNA uptake was analyzed by fluorescent microscopy.

For U937 and NCCIT cells, genomic DNA was purified by ethanol precipitation which eliminates short fragment nucleic acids including unincorporated Cy3 labeled human Cot1 DNA. The relative fluorescent units (RFUs) of Cy3 labeled human Cot1 DNA incorporated into U937 or NCCIT chromosomes were measured using Gemini EM Microplate Reader, and the amount of incorporated human Cot1 DNA was calculated based on Cy3 labeled human Cot1 DNA standard curve.

To model inflammation, all adherent cell lines were activated with lipopolysaccharide (LPS) at 1, 10, and 100 ng per 10^4 cells for 24 to 48 hours in the presence of Cy3 labeled Cot1 DNA fragments. Nuclei were stained with Hoechst stain and then cellular and nuclear DNA uptake was determined by fluorescent microscopy. Additionally, DNA uptake by HFF1 cells was also determined after saponin permeabilization. HFF1 cells were incubated with 0.02% saponin, 300 ng/ml DAPI, and 500 ng Cy3 labeled human Cot 1 DNA for 24, 48, and 72 hours.

Genomics data collection

All currently published autism associated genes (AAGs) were compiled into a single file by combining the AutDB and ACGMap databases.[53,54] Human genomic nucleotide sequences were collected in their entirety by chromosome from UCSC's FTP site, and exon positions for each gene were obtained from UCSC's table browser.[55] Meiotic recombination hotspot locations were retrieved from the International HapMap Project[56] and their coordinates were changed from build 35 to build 37 with the UCSC LiftOver tool (https://genome.ucsc.edu/cgi-bin/hgLiftOver). An algorithm was written to generate all constrained 1024 specific 13 mer sequences and their reverse complements.

Determination of motif and hotspot positions

NCBI's stand-alone BLAST, version 2.2.24, was utilized to locate the positions of all constrained 13 mer hotspot sequences and their reverse complements on each chromosome. Multiple overlaying programs were written to match the 13 mer locations from BLAST with MR hotspot locations on all genes and on the subset of AAGs at both the chromosomal and specific exon levels. These were further utilized to match the hotspot locations and then the 13 mer locations separately within all genes and within the subset of AAGs.

Results

Relationship between MMR coverage and autism prevalence in the United Kingdom, Norway and Sweden after the "Wakefield Scare"

The average MMR coverage for the three countries fell below 90% after Wakefield's 1998 publication but started to recover slowly after 2001 until it reached over 90% coverage again in 2004. MMR coverage is shown by birth year, not year of immunization, explaining why declines in coverage by birth year precede Wakefield's publication since children typically received the MMR vaccination between 12 months and 3 years of age. During the same time period, the average autism disorder prevalence in the United Kingdom, Norway and Sweden dropped substantially for birth year 1998 and gradually increased again after birth year 2000. By 2007, ASD prevalence was twice as high as the prevalence in 1998 (Fig. 1). Autism prevalence and MMR coverage data is shown in Table 1 and 2. While publicly available autism prevalence data for these three countries is disappointingly scarce during this time period, the apparent cause and effect relationship demands further study and investigation.

Residual human fetal DNA quantification and fragment size in Meruvax®II and Havrix®

Average ssDNA and dsDNA in Meruvax®II were 142.05 ng/vial and 35.00 ng/vial respectively, and 276.00 ng/vial and 35.74 ng/vial in Havrix® respectively (Table 3). These amounts are significantly higher than the residual DNA limit <10 ng/dose addressed in the FDA Guidance for Industry.[19] The size of the fetal DNA fragments in Meruvax®II was approximately 215 base pairs by electrophoresis (Fig. 2A). The DNA in Havrix® did not migrate through the gel and stayed in the original well, even when run with lambda phage DNA indicating that the fetal DNA fragments are likely longer than 48,000 base pairs (Fig. 2B).

Human DNA uptake and incorporation in various cell lines

Spontaneous cellular and nuclear DNA uptake was evident in HFF1 (Fig. 3), NCCIT (Fig. 5) and U937 (Fig. 9 and Fig. 10). In contrast, spontaneous Cy3 uptake was not evident in HL-60 cells. DNA uptake in BE (2)-C and M059K was not measurable because of the high autofluorescence of these cells (data not shown). LPS stimulated cellular DNA uptake was observed in HFF1 (Fig. 4), NCCIT (Fig. 6) and M059J (Fig.

7 and 8). Spontaneous and inflammatory enhanced cellular and DNA uptake characterized by quantitating the extent of actual genomic incorporation of the internalized DNA fragments.

The amount of labeled Cy3 human Cot1 DNA incorporation in U937 genomic DNA was 0.0111 ± 0.0034 pg (n=12) per cell within 24 hours, approximately 0.167% of total U937 genomic DNA per cell. DNA incorporation in NCCIT cells was 0.0026 pg/cell after 24 hours and 0.04 pg/cell after 48 hours, an incorporation rate of 0.6% of the total NCCIT genomic DNA (Table 4).

Genome wide double strand break specification signatures

Genomic mapping of MR hotspots has led to the identification of DNA sequence motifs that are overrepresented in hotspots, including a 13 mer degenerate sequence.[29] We found 280,000 occurrences of the identified 13-mer degenerate DNA hotspot motif in the human genome, and of these, the most abundant constrained sequences were CCACCTTGGCCTC and its reverse complement GAGGCCAAGGTGG. Both had recombination rates within the top 30 highest of all 2048 possible constrained 13 mers. This 13 mer hotspot sequence has been associated with 41% of MR hotspots as well as with disease causing nonallelic homologous recombination hotspots and common mitochondrial deletion hotspots.[29]

For reasons unclear at this time, hotspots are concentrated in the subset of autism associated genes (AAGs) and AAGs additionally have some of the highest meiotic recombination rates, in comparison to the set of all genes in the human genome. Compared to all genes, a larger number of AAGs contain MR hotspots, AAGs contain more MR hotspots per gene, and the degenerate 13 mer MR hotspot motif is more prevalent in the hotspots of AAGs than in other genes. While the subset of AAGs compared to all genes in the human genome have similar amounts of degenerate 13 mers per gene, 13 mers are overwhelmingly found more within MR hotspots in the subset of AAGs than in all genes. Specifically, a higher percentage of AAGs contain hotspots, and AAGs have more hotspots per gene, compared to all genes in the human genome (Table 5 and 6). In summary, AAGs have a more concentrated susceptibility for insults to genomic stability in comparison to the group of all genes contained within the human genome. These results further support a disease association between MR, specific constrained 13 mer hotspot motifs and ASD.

Of particular relevance to the male gender bias of ASD is analysis of double strand break specification signatures on the X chromosome. There are 19 autism associated genes on the X chromosome that contain recombination hotspots within transcribed regions. As illustrated in Table 7, 15 of these X chromosome genes have 13 mer motifs less than 100 kilobases away from the center of the hotspot located within an exon. This suggests that these particular X chromosome genes may be particularly susceptible to double strand breaks, insertional DNA mutagenesis and symptomatic disease because 13 mers that are associated with disease have been found near the center of an exon contained hotspot.[29]

Discussion

Vaccine and vaccine safety are a crucial issue for the general public, and in particular for healthcare workers typically required to be fully vaccine compliant as well as for parents who must make informed decisions about vaccinations for their children. The derivation, culture or manufacture of vaccines utilizing electively aborted human fetal tissue poses ethical and philosophical dilemmas. Furthermore, use of human fetal cell lines for the manufacture of vaccines creates final products that contain residual fetal DNA contaminants, including cellular debris and fetal DNA fragments. This poses additional ethical and philosophical dilemmas as these contaminants will be injected into the vaccine recipient along with the virus antigen. And finally, the public was not informed about the fetal cell line vaccine manufacturing, first introduced to the US in 1979, thwarting any public input regarding the ethical and philosophical issues associated with this manufacturing switch.

In addition to the fetal cellular debris and fetal DNA fragments, vaccines that have been cultured on or manufactured using the WI-38 fetal cell line such as Meruvax®II, MMR®II, and Varivax®, are additionally contaminated with fragments of human endogenous retrovirus HERVK.[57] Recent evidence has shown that human endogenous retroviral transcripts are elevated in the brains of patients with schizophrenia or bipolar disorder,[58,59] in peripheral blood mononuclear leucocytes of patients with autism spectrum[60] as well as associated with several autoimmune diseases.[61-63] The public health consequences of the residual human fetal DNA fragments and the HERVK retroviral contaminants have never been adequately studied. We have previously reported an ecological correlation between the introduction of fetal manufactured vaccines and autistic disorder, the first study to consider the public health consequences of manufacturing vaccines in human fetal cell lines.[3]

In this article we have examined the molecular and genomic consequences of fetal DNA fragment containing vaccines, and most importantly presented additional ecological data indicating a potential causal link between fetal manufactured vaccines and the worldwide autism epidemic.[3] After Andrew Wakefield's study was published in the prestigious journal *The Lancet* in 1998 suggesting that MMR®II, a human fetal manufactured vaccine, may be associated with gut abnormalities in autistic children, the debate known as the "Wakefield Scare" was rapidly disseminated through UK and Scandinavian media outlets, leading some parents to choose not to have their children vaccinated with MMR vaccine. The first country affected by the "Wakefield Scare" was the UK according to a paper by Nagaraj in 2006,[8] who stated that "the uptake started declining when the controversial study linking MMR vaccine with autism was published" leading to a very low MMR vaccine coverage as low as 50% in certain areas posing a serious threat of measles epidemics. In Sweden, a drop in MMR coverage in children born after 1999 was reported by Dannetun et al. (2004)[6] because "in 1999 and 2000, there was a widespread public discussion in Sweden on adverse events linked to measles vaccine." In 2002 and 2003, a similar drop in the use of MMR vaccine was reported

for Norway by Trogstad et al. (2012).[7] Similar to the reduction in Sweden, Trogstad et al. (2012) claimed that "this drop coincided with the debate following the false claims about the link between MMR vaccine and autism put forward by Wakefield et al." We illustrate in this publication the abrupt drop of average MMR coverage in the UK, Norway and Sweden following Wakefield's publication and an equally abrupt recovery after 2001 once Wakefield was publicly discredited.

The abrupt drop and recovery in MMR compliance provides a unique opportunity to investigate any causal relationship between fetal manufactured MMR, which was introduced in the UK, Norway and Sweden in 1988, 1983 and 1982 respectively, and ASD prevalence. In order to prove cause and effect, or a causal, relationship rather than a correlation: 1) the cause must occur before the effect, 2) removal of the cause must result in removal of the effect, and 3) reintroduction of the cause must reintroduce the effect. The abrupt drop and recovery in MMR coverage provides a spontaneous real world experiment to examine fetal manufactured vaccine use and ASD prevalence. During the MMR drop and recovery period, ASD prevalence demonstrated a drop after birth year 1998, followed by an upswing that began for birth year 2001 and later. Notably, there is an intriguing association between reductions in MMR coverage and lower AD/ASD rates in Norway, Sweden and UK shortly following Wakefield's 1998 *Lancet* publication.[5] While publicly available ASD prevalence data for these three countries is disappointingly scarce during this time period, the apparent cause and effect relationship as illustrated here demands further study and investigation.

What known biologic processes could explain this apparent causal relationship between fetal manufactured vaccines and ASD prevalence? We suggest that the contaminating fetal cellular debris and DNA fragments as well as HERVK retroviral fragments may lead to autoimmune attack and/or insertional mutagenesis in children. This paper focuses on the potential for insertional mutagenesis.

In this article we report the presence of DNA fragments in Meruvax®II and Havrix®. As shown in Figure 2, the residuals in Meruvax®II are approximately 215 base pairs in length. The size of residual DNA contents in Havrix® was not measureable. Our results demonstrate the excessive levels of ssDNA and dsDNA in Meruvax®II and Havrix®, which are significantly higher than the FDA guidance suggested limit.[19] These excessive residuals can be taken up by a cell and delivered to the nucleus. The potential for exogenous DNA to enter the nucleus of a cell and insert into the genome of that cell is a well-established biologic process. Indeed, as mentioned earlier, nuclear transfer of mitochondrial DNA fragments is an ongoing process in mammals, including humans.[64] Efforts to develop gene therapy have documented that short DNA fragments can and do insert into a recipient's genome with an efficiency of up to 20%. Yakubov et al. (2007) utilized human placental DNA fragments of 200 to 3000 base pairs in length and demonstrated spontaneous uptake and insertion into the genome of MCF-7 human cell line of 1-4% efficiency, and also demonstrated the species specific nature of this genomic incorporation because fragments of salmon sperm DNA did not integrate into

the human MCF-7 cells.[18] In this study we report similar genomic incorporation rates of 0.2 to 0.6% of recipient genome 24 to 48 hours after addition of DNA fragments of approximately 350 base pairs in length to the culture media of U937 or NCCIT cells. DNA fragment uptake was spontaneous and did not require cell permeabilization or transfection. Inflamatory stimuli (LPS) were able to enhance the internalization, indicating the importance of a child's general health at the time of vaccination for susceptibility to internalize the injected fetal and retroviral fragments.

Most significantly, small fragments of DNA have also been shown to integrate into the genome efficiently *in vivo*. Jensen et al. (2011) have successfully delivered and integrated small DNA fragments to the mouse liver using tail vein injections for administration.[65] Colosimo's studies in 2001 and 2007 also demonstrated 1-10% *in vivo* gene integration of small homologous fragments in 4% of CFTR mutant human epithelial cells.[66,67] McNeer et al. (2013) delivered DNA fragments up to 60 base pairs in length to mice by tail vein injection, and demonstrated 0.01 to 0.04% genomic integration with DNA alone that was increased to up to 1.2% with triplex forming PNA. Genomic integration was highest in the cells found in the bone marrow, spleen and thymus.[68] Furthermore, using these same techniques, McNeer et al. (2013) have also demonstrated *in vivo* DNA targeting efficiencies, using systemic intravenous injection, of between 0.05% and 0.43% using deep gene sequencing of bone marrow and spleen cells respectively. McNeer et al. (2013) also demonstrated the species specific nature of DNA integration in that mice chimeric for human CD34+ stem cells containing a CCR5 mutation showed uptake of the injected fragment only in the human CD34+ stem cells but not in the mouse CD34+ stem cells in these chimeric animals.[68]

Cell free DNA can be taken up by healthy cells via receptor mediated uptake or may spontaneously penetrate cell membranes that have altered permeability, for instance, during inflammatory reactions. Nuclear uptake of cell free DNA fragments is thought to provide a source for maintenance of DNA integrity during rescue of collapsed replication forks or base lesion repair. Spontaneous extracellular DNA uptake has also been exploited for gene therapy as well as for cellular gene correction.[9,18,69-72] While free DNA uptake has been used advantageously, the process has also been associated with the generation of mutations and chromosomal aberrations.[73] Our measured genomic incorporation (0.003 to 0.04 picograms) of 0.2% - 0.6% of the whole genome in 24 to 48 hours seems high at first glance. However, our numbers are consistent with previous reports showing that exogenous DNA replaced up to 1% of the whole genome within 30 minutes.[9] Although HL-60 cells did not spontaneously take up exogenous DNA in our experiments, the cell line has been used in the past as a model for spontaneous DNA uptake.[72] Cellular and nuclear DNA uptake in HFF1 cells and in NCCIT cells suggests that embryonic and neonatal cells are more susceptible to DNA uptake than cells from a more mature source. These results indicate the need for further study of DNA incorporation from exogenous sources to compare the susceptibility of infants and toddlers versus teens and adults. Furthermore, increased DNA uptake after LPS activation suggests

that systemic inflammation or immune responses could increase the susceptibility for exogenous DNA uptake. Vaccines that are manufactured using human fetal cell lines are contaminated by exogenous DNA and retroviral fragments,[57] and vaccines elicit systemic inflammation and immune activation, a combination that may be particularly amenable to insertional mutagenesis in vaccine recipients.

In addition to confirming previous reports on DNA incorporation in human cells, the genomic analysis in this current paper indicates that there are regions in the disease free genotype in which mutations that could trigger ASD are likely to occur (Tables 5 and 6) when considering genomic susceptibility to develop ASD in terms of DSB specification signatures alone. What might account for the higher rate of 13 mers (DSB specification signatures) in AAGs? It has been demonstrated by other researchers that AAGs are characteristically hypermutable,[74] which may be the result of the high concentration of DSB specification signatures in these genes. As far as actual symptomatic ASD, some factor in addition to programmed DSB formation could easily manifest and lead to de novo mutations, such as induced DSBs in somatic cells, and this study suggests that fetal DNA fragments in vaccines may be such an environmental factor. In a similar scenario as is the case of various lymphomas, for example, the addition of a toxin or chemotherapeutic induced DSB to programmed DSBs for class switching leads to the cancer.[30]

In summary, vaccines manufactured in human fetal cell lines contain unacceptably high levels of fetal DNA fragment contaminants. Human DNA fragments of similar length and epigenetic signature spontaneously integrate into the genome of primitive cell lines, a process that can be augmented in the setting of inflammation. The human genome naturally contains regions that are susceptible to double strand break formation and DNA insertional mutagenesis, regions that are particularly concentrated within the exons of genes that have been shown to be causative or associated with ASD phenotype. The 'Wakefield Scare' created a natural experiment that indicates a causal relationship between fetal cell-line manufactured vaccines and ASD prevalence. Our paper calls for additional study and investigation of this potential relationship. A solution to this horrific public health problem is readily available: vaccines can be safely and effectively manufactured in animal, insect or plant based cell lines,[75-77] eliminating the dangers of residual human DNA and retroviral contaminants.

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Figure 1

Autism prevalence and MMR coverage in the UK, Norway and Sweden before and after Wakefield's 1998 paper suggesting MMR was linked to bowel disease and autism

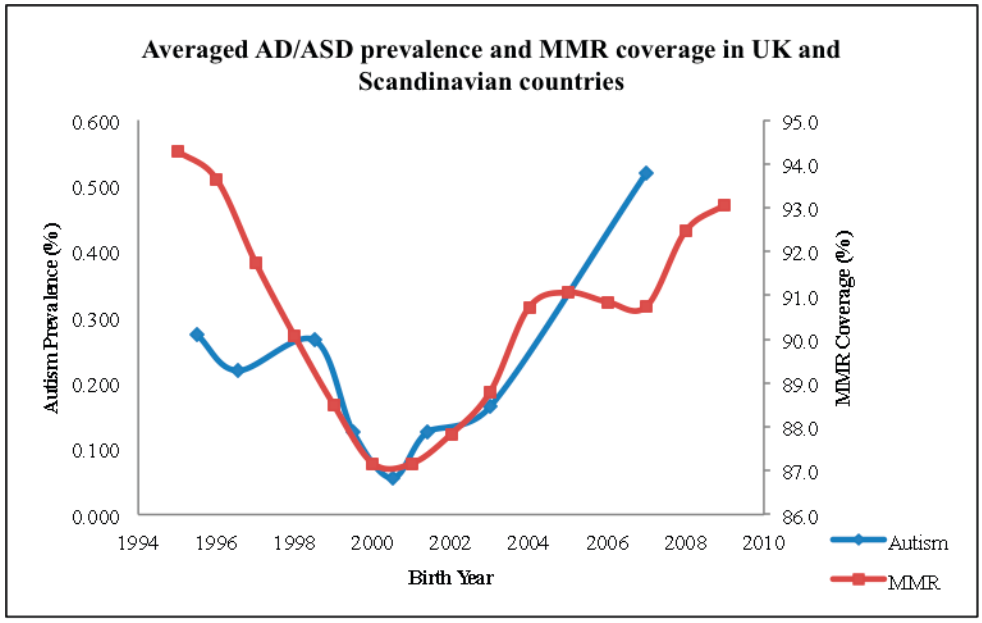

Figure 1-Averaged AD/ASD prevalence and MMR coverage in UK, Norway and Sweden. Both MMR and AD/ASD data are normalized to the maximum coverage/prevalence during the time period of this analysis.

Table 1

Autism Prevalence in Norway, United Kingdom and Sweden

Birth Year	Prevalence (%)	
	Norway	
1999	0.14	Isaksen et al. 2012 Suren 2012 & 2013
2000	0.05	
2001	0.17	
2002	0.26	
2003	0.29	
	United Kingdom	
1995.5	0.274	Latif et al. 2007 Lingam et al. 2003
1996.5	0.219	
1998.5	0.265	
1999.5	0.110	
2000.5	0.061	
2001.4	0.082	
	Sweden	
1998	0.12	Nygren et al. 2012
2003	0.038	
2007	0.52	

Table 2

Averaged MMR Coverage in United Kingdom, Norway and Sweden

Birth Year	MMR Coverage (%)
1995	94.3
1996	93.7
1997	91.8
1998	90.1
1999	88.5
2000	87.2
2001	87.2
2002	87.8
2003	88.8
2004	90.7
2005	91.1
2006	90.9
2007	90.8
2008	92.5
2009	93.1

Table 3

Levels of residual human single stranded DNA (PicoGreen® assay) and human double stranded DNA (OliGreen® assay) in Rubella vaccine (Meruvax®II) and Hepatitis A vaccine (Havrix®).

	MERUVAX®II (Rubella vaccine)		HAVRIX® (Hepatitis A vaccine)	
	Merck & Co. Inc.		***GlaxoSmithKline Biologicals***	
	Single stranded DNA (ng/vial)	Double Stranded DNA (ng/vial)	Single stranded DNA (ng/vial)	Double Stranded DNA (ng/vial)
Vial 1	135.66	59.34	844.58	112.69
Vial 2	145.82	24.54	104.88	10.69
Vial 3	-	36.052	213.6	15.32
Vial 4	-	20.064	213.6	15.32
Vial 5	118.54	-	213.6	15.32
Vial 6	164.40	-	165.43	73.33
Vial 7	145.82	-	176.31	7.49
Average	142.05	35.00	276.00	35.74

Figure 2

Purified DNA after gel electrophoresis. Representative gels showing residual human DNA fragments size for Meruvax®II (A) and Havrix® (B).

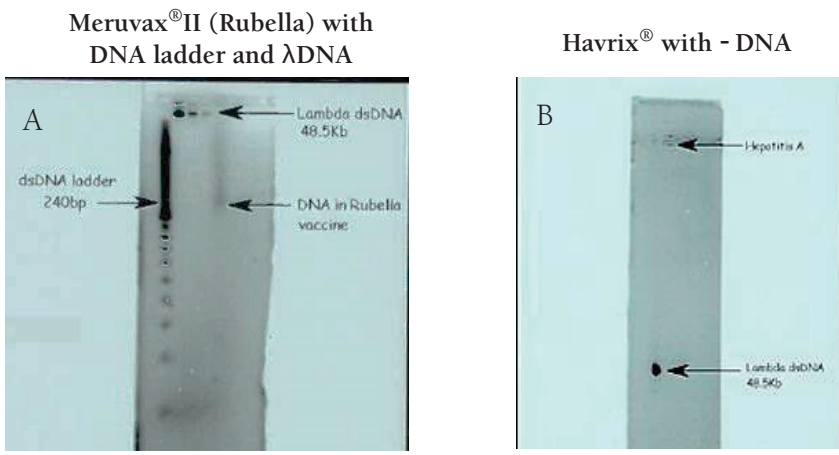

Figure 2 – Human fetal DNA fragment size in Meruvax®II averaged 215 base pairs in length but ranged from 700 base pairs down to 200 base pairs in length (2A). When run under identical conditions as for 2A, human fetal DNA fragments in Havrix® did not move out of the well. Additional gels were run for extended period of time following lambda DNA migration through the gels and still human fetal DNA fragments in Havrix® did not move out of the well (2B).

Table 4

Cy3 labeled human Cot1 DNA uptake in various cell lines.

	Spontaneous Cellular uptake	Spontaneous Nuclear uptake	Incorporation in Genomic DNA	Cellular /Nuclear Uptake with LPS or saponin
HFF1	Yes	Yes	Not Done	Increase/Increase
NCCIT	Yes	Yes (variable)	0.0026 pg per cell 24 0.04 pg per cell 48	Same/Same
BE(2)-C	No	No	Not Done	No/No
M059K	No	No	No	No/No
M059J	No	No	Not Done	Yes/No
U937	Yes	Yes	0.011 ± 0.003 pg per cell 24	Same/Same
HL60	No	No	No	No

Table 4 – Human Cot1 DNA purchased from Invitrogen with an average fragment size of 350 base pairs (data not shown), was labeled with Cy3 and incubated with various human cell-lines. Cellular, nuclear, and genomic uptake was followed using fluorescence microscopy and fluorometry. Uptake was determined under resting as well as activated conditions.

Figures 3 through 10

Cy3 labeled human Cot1 DNA fragment uptake in various human cell-lines representing embryonic, neonatal and cancer transformation stages.

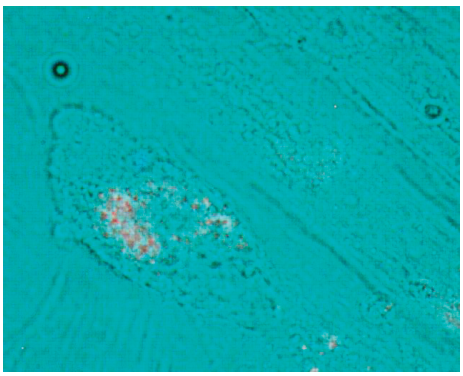

Figure 3 - HFF1 spontaneous cellular and nuclear DNA uptake (bright field & Cy3 red fluorescence overlay).

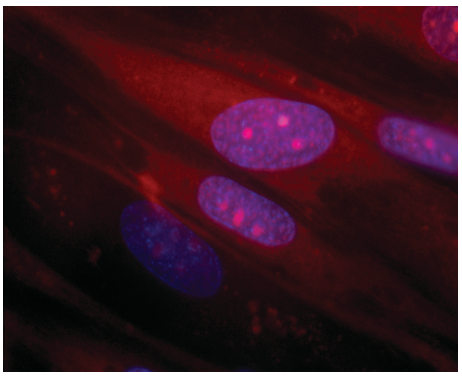

Figure 4 - HFF1 cellular and nuclear DNA uptake after permeabilization with saponin. (Cy3 red & nucleus Hoechst blue fluorescence overlay)

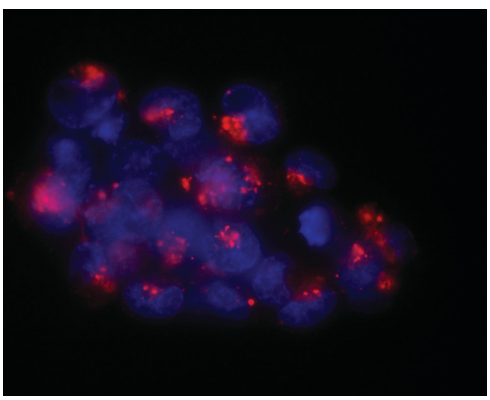

Figure 5 - NCCIT spontaneous cellular DNA uptake (Cy3 red & nucleus blue fluorescence overlay)

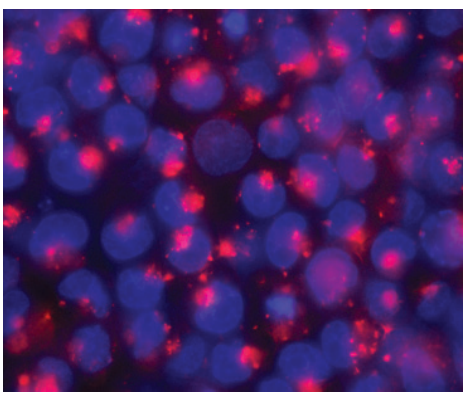

Figure 6 - NCCIT cellular DNA uptake after lipopolysaccharide activation (Cy3 red & nucleus Hoechst blue fluorescence overlay)

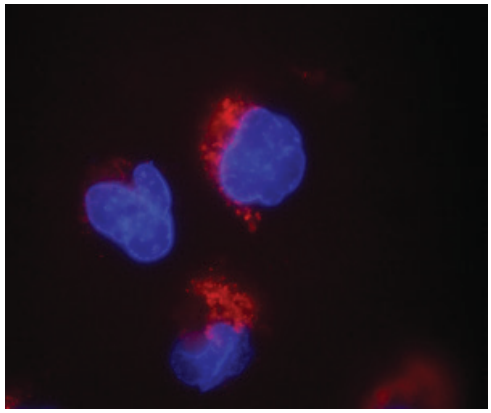

Figure 7 - M059J cellular DNA uptake after lipopolysaccharide activation (10 ng/10^4 cells) (Cy3 red & nucleus Hoechst blue fluorescence overlay).

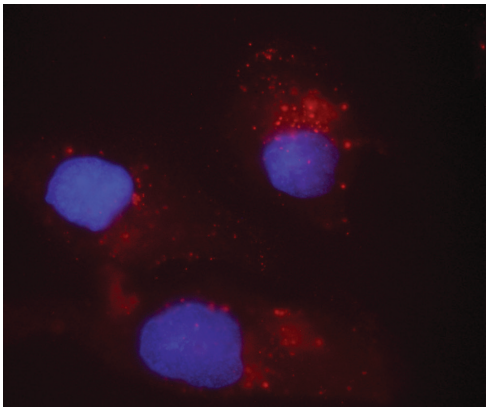

Figure 8 - M059J cellular DNA uptake after lipopolysaccharide activation (100 ng/10^4 cells). (Cy3 red & nucleus Hoechst blue fluorescence overlay).

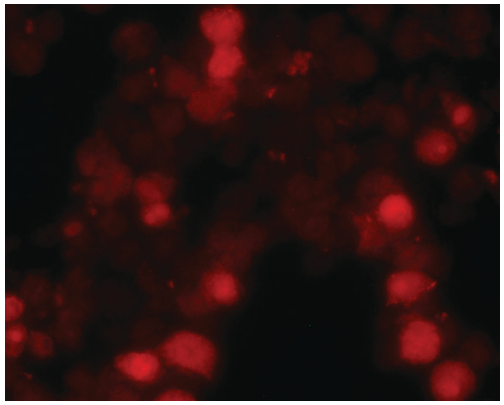

Figure 9 - U937 spontaneous cellular/ nuclear DNA uptake (Cy3 red)

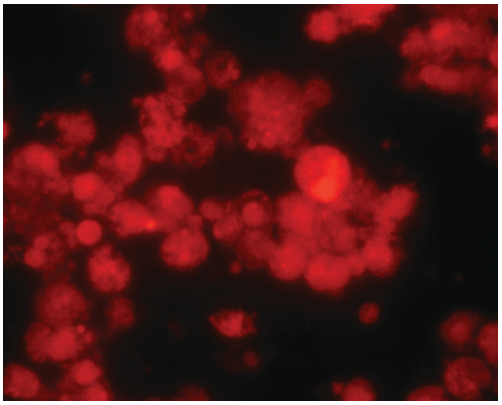

Figure 10 - Purified U937 nuclei containing Cy3 labeled DNA before DNA purification (Cy3 Red)

Table 5

Comparison of chromosomal average hot spots and 13 mer concentrations

	Hotspots per Gene	13 mers per Gene	13 mers in Hotspot per Hotspot
All Genes	2.38	16.8	1.63
AAGS	4.21	17.7	2.22

Table 6

Comparison of 13 mer presence within all genes and autism associated genes

Chromosome	% Genes Containing HS		% Genes Containing 13 mer in HS		% Genes Containing 13 mer	
	All genes	**AAGS**	**All genes**	**AAGS**	**All genes**	**AAGS**
1	23.42%	50.00%	13.05%	27.78%	72.97%	72.22%
2	16.97%	62.50%	16.97%	45.83%	78.14%	95.83%
3	27.56%	60.87%	14.99%	52.17%	79.94%	86.96%
4	35.24%	50.00%	17.62%	32.14%	72.17%	78.57%
5	28.68%	36.67%	16.32%	20.00%	75.27%	83.33%
6	26.15%	33.33%	12.70%	23.33%	71.85%	83.33%
7	25.52%	63.00%	18.78%	54.00%	77.00%	97.00%
8	28.95%	53.85%	16.42%	30.77%	74.70%	92.31%
9	23.20%	75.00%	14.42%	62.50%	72.10%	75.00%
10	31.63%	60.00%	17.28%	40.00%	79.78%	90.00%
11	17.85%	36.00%	9.38%	24.00%	65.83%	92.00%
12	20.12%	37.50%	12.59%	37.50%	77.40%	100.00%
13	37.65%	77.78%	20.05%	66.67%	74.57%	88.89%
14	26.49%	66.67%	16.41%	66.67%	64.34%	100.00%
15	23.57%	46.67%	11.79%	46.67%	68.04%	86.67%
16	17.81%	46.15%	10.75%	38.46%	76.61%	92.31%
17	17.10%	26.67%	12.51%	20.00%	74.24%	100.00%
18	32.53%	0.00%	19.88%	0.00%	79.52%	100.00%
19	10.11%	0.00%	8.06%	0.00%	76.38%	100.00%
20	23.88%	44.44%	15.25%	33.33%	74.58%	77.78%
21	25.00%	100.00%	20.42%	100.00%	69.00%	100.00%
22	17.65%	11.11%	13.24%	11.11%	79.78%	100.00%
X	14.00%	45.00%	13.45%	39.00%	65.00%	95.00%
Average	**24.43%**	**47.10%**	**14.95%**	**37.91%**	**74.28%**	**90.75%**

Table 7

X Chromosome AAGs with 13 mers in exons and their distance from the closest hotspot (HS) start

Gene	Gene Start	Gene End	Motif Start	Motif End	Motif	Strand	Exon Start	Exon End	HS Start	HS End	Distance
NLGN4X	5808082	6146706	5821352	5821364	gtggccacggcgg	-	5821117	5821907	5811910	5813910	7442
AFF2	147582138	148082193	148048477	148048489	ccaccatcacctc	+	148048319	148048609	148036319	148040319	8158
NLGN3	70364680	70391051	70386983	70386995	gtggatatggtgg	+	70386860	70387650	70371089	70377089	9894
MECP2	153287263	153363188	153296166	153296178	gtggtgatggtgg	-	153295685	153296901	153306976	153308976	10798
FRMPD4	12156584	12742642	12739916	12739928	ccaccatggccgc	+	12738647	12742642	12721454	12727454	12462
PHF8	53963112	54071569	54012352	54012364	ccaccatgtcctc	-	54012339	54012382	54025089	54033089	12725
RAB39B	154487525	154493852	154493567	154493579	cctccatggccgc	-	154493358	154493852	154478566	154480566	13001
FRMPD4	12156584	12742642	12736309	12736321	ggggcaagggagg	+	12735619	12736909	12721454	12727454	14867
GRPR	16141423	16171641	16170710	16170722	cctccgtggccac	+	16170378	16171641	16208454	16211454	37732
AFF2	147582138	148082193	148079262	148079274	cccccgtcaccac	+	148072740	148082193	148036319	148040319	38943
SH3KBP1	19552082	19905744	19564111	19564123	cctccttatcctc	-	19564039	19564168	19610454	19614454	46331
PDZD4	153067622	153096003	153070331	153070343	cccccttctcctc	-	153067622	153070355	153013976	153018976	51355
SLC6A8	152953751	152962048	152960611	152960623	ccaccctgacccc	+	152960528	152962048	153013976	153018976	53353
FMR1	146993468	147032647	147026533	147026545	gaggacaaggagg	+	147026463	147026571	147083193	147085193	56648
HNRNPH2	100663120	100669128	100668241	100668253	gtggttatggtgg	+	100666923	100669128	100725023	100727023	56770
HNRNPH2	100663120	100669128	100668229	100668241	gtggttatggagg	+	100666923	100669128	100725023	100727023	56782
HNRNPH2	100663120	100669128	100667689	100667701	gagggtatggagg	+	100666923	100669128	100725023	100727023	57322
FMR1	146993468	147032647	146993528	146993540	gcggtgacggagg	+	146990948	146993748	147083193	147085193	89653
CXCR3	70835765	70838367	70837189	70837201	gtgggcagggcgg	-	70835765	70837309	71057089	71064089	219888
AR	66763873	66944119	66765398	66765410	ccgccgtggccgc	+	66763873	66766604	68319089	68321089	1553679