The Opa protein repertoires of disease-causing and carried meningococci.


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Keywords: Neisseria meningitidis, Opa, Severity, MLST

Running title: Meningococcal Opa proteins
Abstract

The meningococcal Opa proteins play an important role in pathogenesis by mediating invasion of human cells. The aim of this investigation was to determine whether carried and disease-associated meningococci possess different Opa repertoires and whether the diversity of these proteins is associated with the clinical severity of disease. Opa repertoires in 227 disease-associated meningococci, isolated in the UK over a period of 7 years, were compared to the repertoires in 190 asymptptomatically carried meningococci isolated in the UK from a contemporary, non-epidemic period. Multidimensional scaling (MDS) was employed to investigate the association between Opa repertoires and MLST genotypes. Associations with clinical severity were also analysed statistically. High levels of diversity were observed in opa alleles, variable regions and repertoires, and MDS revealed that MLST genotypes were strongly associated with particular Opa repertoires. Individual Opa proteins or repertoires were not associated with clinical severity, though there was a trend towards an association with the opaD locus. The meningococcal Opa repertoire is strongly linked to MLST genotype irrespective of epidemiological sampling and therefore correlates with invasiveness. It is not, however, strongly associated with the severity of meningococcal disease.
Introduction

Invasive meningococcal disease caused by the Gram negative bacterium *Neisseria meningitidis* (the meningococcus) has an incidence of 1-6 cases per 100,000 population in Europe (40) and a mortality rate of approximately 8% (42). The disease is associated with different clinical presentations, most commonly meningitis or septicaemia, or a combination of the two. Its clinical severity is likely to be dependent on host and bacterial factors that influence the inflammatory and immunological responses of the host (9, 12). A variety of meningococcal surface and secreted proteins play roles in contact with host cells (30) and some of these regulate host immunological responses (1, 35). Few studies have investigated the distribution of the proteins involved in these interactions in the meningococcal population or their effects on the clinical severity of invasive disease.

The opacity associated adhesin (Opa) proteins (Figure 1), located on the meningococcal surface, promote intimate interaction with the host (44) and modulate host immunological responses. Topologically, Opa proteins display an 8-stranded transmembrane β-barrel structure with 4 extramembranous putative loop regions (7). A semivariable (SV) and two hypervariable regions (HV1 and HV2) are present in three of the loop regions and a large number of Opa variants have been observed (5, 22). Sequence variation, particularly in the two hypervariable regions, influences the specificity of Opa variants for different members of the human carcinoembryonic antigen cell adhesion molecule (CEACAM) family of proteins (45, 46) and cell-surface saccharides (26). These interactions lead to adhesion between the bacterium and host, or adhesion followed by invasion of the host cells dependent on the particular Opa variants and receptors involved (28, 43). The Opa proteins of *Neisseria*...
species also modulate the host immune response through interactions with cells of the immune system, including neutrophils and CD4+T cells(16, 24, 45). Opa proteins also stimulate the secretion of proinflammatory cytokines, the levels of which correlate with severity of meningococcal disease(4, 31), from human macrophages(21).

The Opa proteins are encoded by four genetic opa loci in each isolate(32, 39), encoding its Opa repertoire. Population-based studies of Opa diversity have suggested that the meningococcal hyperinvasive clonal complexes, genotypes identified by multilocus sequence typing (MLST) responsible for the majority of global meningococcal disease(14, 20), are associated with particular Opa repertoires(5, 27). It is unclear whether this association is unique to disease-causing meningococci or whether clonal complexes isolated from asymptomatic carriage that have never, or rarely, caused disease also exhibit this feature. Furthermore, it is unclear whether these combinations in disease-causing meningococci are associated with differences in clinical severity.

The influence of Opa variation on the severity of meningococcal disease, potentially through stimulation of proinflammatory cytokine production or through increases in bacterial invasiveness mediated by different Opa protein variants, is unclear due to the lack of information on the function and frequency distribution of Opa variants among meningococci. The aim of this investigation was to compare the opa gene repertoires of meningococci isolated from invasive disease with asymptptomatically carried meningococci collected during a contemporary, non-epidemic period and to determine whether opa diversity is associated with the clinical severity of meningococcal disease.
Materials and methods

Meningococcal isolate collection: A total of 417 *N. meningitidis* isolates were analysed in this investigation, obtained from both asymptomatic carriage and invasive meningococcal disease. This was a largely contemporaneous collection, with all carriage isolates collected during 1999 and disease isolates collected during the 7 years between 1996 to 2001. All isolates were from the UK, but most were from the South East of England. The isolates are described more fully below.

A set of 190 isolates were representative of a larger number of isolates collected from individuals carrying the bacterium in Oxfordshire, a county in the South East of the England, UK, during 1999. Nasopharyngeal swab samples were taken from 15-19 year old students attending school or college who were recruited as previously described(19), with informed consent obtained in writing. The study was approved by the Trent MultiCentre Research Ethics Committee (study number: MREC/99/4/036).

Meningococci were identified by positive culture and have been characterised by MLST(18).

A further 227 isolates from invasive meningococcal disease were obtained from two sources of patients. A total of 120 isolates were obtained following admission to the paediatric intensive care unit at St. Mary’s Hospital, London, UK, approximately 50 miles (~80km) from Oxford, between 1992 and 2002. A further 107 isolates were collected as part of a national UK meningococcal disease study overseen by the Royal College of Paediatrics and Child Health (RCPCH) in which all fatal paediatric meningococcal disease cases between December 1st, 1997 and February 28th, 1999 were investigated. The severity data and disease isolates used in the current study
were collected and analysed under St. Mary’s Hospital Local Research Ethics Committee approval number EC3263. This isolate collection was originally assembled for analysis as part of the European Meningococcal Monitoring Network (EUMenNet)(10, 41). Full multilocus sequence typing (MLST) analysis of isolates from disease isolates will be described elsewhere.

For 182 out of the 227 disease isolates, a Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS) of clinical severity(37) was assigned retrospectively from case notes. The GMSPS is a widely used, simple, clinical scoring system based on 7 clinical parameters including degree of hypotension, skin/rectal temperature difference, base deficit, extent of hemorrhagic skin lesions, the Simpson and Reilly paediatric coma score, presence of meningism and the parents’ opinion of any change in the patient’s condition in the hour prior to scoring. A score of 8 or above out of a total of 15, has 100% sensitivity, 75% specificity and 29% positive predictive value for death, the risk of which increases as the score rises above 8(34).

Preparation of meningococcal genomic DNA: Isolates were revived from storage below -70°C by growth on single plates of Columbia agar in a 5% CO2 atmosphere, at 37°C for 18 hours or overnight. Meningococcal growth from each individual plate was collected into phosphate buffered saline solution (1ml) and heated to 56°C to kill the bacteria before genomic DNA was extracted using a Qiagen DNA mini kit (Qiagen) according to the manufacturers instructions. Purified genomic DNA was solubilised in distilled/deionised water and stored in below -20°C.
Determination of opa gene nucleotide sequences: The meningococcal Opa proteins are encoded by 3-4 phase variable, unlinked genomic opa loci (opaA, opaB, opaD and opaJ)(32, 39). As previously described(5), the nucleotide sequence of each opa locus was determined. Briefly, each opa locus was amplified by PCR using locus-specific primer sets and nucleotide sequences were determined directly from PCR products. Variable region amino acid sequence variants, and sequence variants within families, were identified by comparison with previously published data and new sequence variants/variant families were assigned arbitrarily in the order of discovery, all according to previously published nomenclature(5). New opa alleles have been posted in Genbank.

Alignments of amino acid sequences and the original nucleotide sequences were created using the CLUSTALW algorithm in the program DAMBE(47). Nucleotide and amino acid p-distances were calculated using the MEGA v.3.0 software package(15), compensating for sequence alignment gaps using a pairwise deletion method. The Opa repertoires were described by the combination of HV1 and HV2 amino acid sequence families at each locus in the same isolate, with each unique HV1-HV2 combination, and each unique repertoire of these combinations, assigned an arbitrary identification number. This approach reduced the high diversity observed at the allelic level, allowing identification of relationships among repertoires comprising different alleles encoding identical protein sequences. Furthermore, this approach allowed the analyses to focus on the Opa HV region sequences and their combinations, which contain functionally-important receptor binding sequences.
Analysis of Opa repertoire clustering: Classical multidimensional scaling (MDS) analysis(17, 33) was employed to investigate relationships among Opa repertoires as defined by HV1 and HV2 sequence variants (i.e. the functionally and antigenically important regions of Opa) at all four loci. First, the differences in the Opa repertoires among isolates were determined by comparing each isolate to every other and counting the number of different HV region variants between each pair of isolates (with 8 being the maximum number different from a total of four HV1 and four HV2 variants). Identical HV regions occurring at multiple opa loci in a pair of isolates were treated as equal, and loci where an opa allele was not detected were treated as different. The resulting ‘distance matrix’ was then used as input for the MDS algorithm. The algorithm determined the relationships among isolates by displaying the distance matrix as a set of data points, with one data point for each isolate that best represented its position in relation to other isolates in Euclidean geometric space. This was represented as a multidimensional scatter plot. The number of spatial dimensions in the MDS analysis was allowed to vary, but the lowest number of dimensions that described the largest proportion of the data was defined as the most efficient at depicting the relationships among isolates based on their Opa repertoires.

Statistical analysis of the association between Opa repertoires and clinical severity of meningococcal disease: The overall association between Opa and severity was assessed by correlation between distance matrices derived from the Opa repertoires and a distance matrix based on GMSPS scores. These were compared using a Mantel test and the correlation tested using a 1000 iteration permutation test as implemented in the program ARLEQUIN(36). The association between GMSPS and opa alleles or Opa variable region amino acid sequence families was also assessed using linear
regression with GMSPS as the outcome variable and *opa* alleles or Opa variable region amino acid families as a categorical independent variable. Statistical significance was assessed by F-tests.
Results

*opa diversity in meningococci isolated from cases of invasive disease:* Among the 227 isolates from meningococcal disease analysed in this investigation, *opa* gene sequences were detected in 833 of the total 908 loci examined (92%), encoding 205 different *opa* alleles (Table 1). Meningococci have 3-4 *opa* loci and in isolates from the invasive disease collection, alleles were not detected at one *opaA* locus, one *opaB* locus, at the *opaD* locus of 29 isolates, one of which had a frameshift mutation and 44 *opaJ* loci, all of which were disrupted by insertion sequence-like elements. PCR and sequencing were repeated with new genomic DNA preparations, alternative primers and cycling conditions for loci at which an *opa* allele could not be detected. The 205 alleles encoded 55 HV1-HV2 sequence family combinations (Figure 2), of which 12 were present above 2% frequency, accounting for 705 (85%) of the 833 *opa* loci. The single most common HV1-HV2 family combination was composed of HV1 family 11 and HV2 family 1 (combination 40), present at 93 loci, of which 82 were present at the *opaD* locus of ST-11 and ST-8 complex isolates. Other occurrences included the *opaA* locus of 8 ST-11 isolates with these variable regions also present at their *opaD* loci.

*opa diversity in meningococci isolated from nasopharyngeal carriage:* Among the 190 meningococcal carriage isolates, 246 different *opa* allele sequences were detected in 684 (90%) of the total 760 loci analysed (Table 2). A full length *opa* allele was not detected at the *opaA* locus 14 isolates, two of which were disrupted by frameshift mutations, at the *opaB* locus of seven isolates, at the *opaD* locus of 25 isolates and at the *opaJ* of 30 isolates, two of which were disrupted by frameshift mutations. A further three isolates had alleles at their *opaJ* locus that were highly similar by
alignment to opa genes from Neisseria lactamica (data not shown). These alleles were identical in the isolates 99-30740 and 99-30928, both members of the ST-198 clonal complex. As in the collection of disease isolates, PCR and sequencing was repeated with new genomic DNA preparations, alternative primers and cycling conditions for isolates at which an opa allele was not detected. There were 65 combinations of HV1-HV2 families (Figure 2) with 15 combinations present above 2% frequency, accounting for 555 (81%) of loci. The single most common HV1-HV2 family combination was combination 75, composed of HV1 family 19 and HV2 family 14, present at 66 loci, equating to 10% of total loci, appearing in isolates belonging to multiple clonal complexes.

Comparison of Opa repertoires in carried meningococci versus disease-related isolates: There were a total of 269 Opa repertoires in the 417 meningococci analysed, based on the combination of HV1 and HV2 families at each of the four loci per isolate. In isolates from meningococcal disease there were 133 Opa repertoires (based on the combination of HV1 and HV2 sequence families at each locus), 16 of which were present above 1% frequency (appearing in more than 3 isolates). The most frequent repertoire was composed of HV1-HV2 combinations OpA: 27, OpB: 69, OpD: 40 with the opaJ locus inactivated due to the presence of an insertion sequence element. This repertoire was observed in 39 (17%) of isolates from invasive disease and all of these were members of the ST-11 complex and was identical to that of carried and disease-associated ST-11 meningococci responsible for an epidemic in the Czech Republic in 1993 (Callaghan and Buckee et al. in preparation). In carried meningococci there were 142 repertoires, of which 25 were observed above 1% frequency (appearing in more than 2 isolates). The most common of these was
characterised by HV1-HV2 combinations OpaA: 67, OpaB: 72 and OpaJ: 40. An opa
gene was not detected at the opaD locus in these isolates. This repertoire was present
in 10 meningococci, 9 of which were members of the ST-53 complex. A total of five
repertoires were present in both collections at low frequencies, appearing in no more
than 9 disease isolates and 2 carriage isolates.

Structuring of the Opa repertoires of individual clonal complexes: Analysis of the
Opa repertoires of individual clonal complexes in the combined collection of carriage
and disease isolates indicated that the Opa repertoires of some complexes were more
conserved, composed of fewer Opa proteins, than others. The three most common
clonal complexes were the ST-11, ST41/44 and ST-269 complexes, whose repertoires
were composed of 6 or 7 Opa proteins in each complex, with 3-4 variants above 10%
accounting for 71% of the total number of loci analysed in all isolates from the ST-11
complex (HV1-HV2 combinations 27, 40, 69), 62% of the total loci in all isolates of
the ST-41/44 complex (HV1-HV2 combinations 4, 11, 20, 31) and 59% of the total
loci in all isolates of the ST-269 complex (HV1-HV2 combinations 59, 61 and 69).
No differences in the Opa repertoires of individual clonal complexes between
asymptomatic carriage and invasive disease were apparent.

MDS analysis revealed that Opa repertoires of genetically related isolates clustered
together (Figure 3) irrespective of whether they were isolated from disease or
carriage. Four major clusters were observed, each containing disease-related and
carried isolates (Figure 4). Two of these clusters were comprised mostly of disease-
related isolates, one containing the majority of isolates belonging to the ST-41/44
complex whereas the other included members of the ST-11 and ST-269 complexes.
The variance of the scattering of repertoires was larger in isolates from disease, with data points distributed further apart (mean variance in the three dimensions shown was 3.051), whereas the data points for repertoires from carried isolates clustered tighter (mean variance in the three dimensions shown was 2.079). The spread of data points along the three axes also differed between disease and carried isolate repertoires. Values for carried isolates were x: 1.777, y: 1.6499 and z: 2.809. In disease isolates, the values were x: 5.889, y: 2.165 and z: 1.098. These data indicated that the repertoires of disease isolates mostly clustered along the x axis, whereas those of carried isolates tended towards the y axis.

Association of Opa diversity and clinical severity: GMSPS data was available for 182 patients of the 227 for whom meningococcal isolates were collected (80.2%). Of these, 96 out of 182 (52.75%) had a score of 0-7 whereas 86 of 182 (47.25%) had scores of 8 or higher. The null hypothesis, that the opa gene or Opa protein repertoire of meningococci is not associated with clinical severity of meningococcal disease, was investigated. No evidence for a correlation between the distance matrices constructed from the Opa repertoire (HV1-HV2 family combinations) and the severity scores was found (correlation coefficient of –0.001 and p-value of 0.51). There was no significant relationship observed between any component of opa alleles or Opa proteins and severity as measured by the GMSPS. A trend was observed, however, for an association across the data set between the three variable regions encoded by alleles at the opaD locus and severity, with p values of approximately 0.1 (data not shown).
Discussion

The data presented here show that meningococcal Opa repertoires and MLST genotypes are closely linked, irrespective of epidemiological sampling. Isolates from carried genotypes, as well as those from hyperinvasive genotypes, had highly structured, similar Opa repertoires. An association between Opa diversity and the clinical severity of meningococcal disease was not found, although there was a trend towards an association at the \textit{opaD} locus.

MDS analysis of the diversity of the Opa repertoires in this investigation indicated an association with meningococcal MLST sequence types and clonal complexes. Previous studies have identified combinations of Opa proteins consistently observed in meningococcal hyperinvasive clonal complexes over decades of epidemic spread\cite{5, 27}. Hyperinvasive clonal complexes are not representative of the genetic diversity observed in asymptomatically carried meningococcal populations however, which are composed of numerous, diverse genotypes\cite{13, 14}. Consequently, the association between Opa repertoire and MLST genotype cannot be extended to carriage populations by inferences made using data solely from hyperinvasive complexes. In this investigation the asymptomatically carried meningococci were all genetically diverse serogroup B and C isolates, collected from asymptomatic carriers during a contemporary, non-epidemic period in the UK. Furthermore, the majority of these isolates belonged to clonal complexes that are never, or have rarely been, isolated from disease. These data now provide additional evidence that meningococcal isolates belonging to the same sequence type or clonal complex are consistently associated with particular combinations of Opa adhesins. This association appears to be epidemiologically stable, maintained whether isolates are collected from carriage or
disease. Furthermore, MDS analysis supported a trend towards differential clustering of Opa repertoires in meningococci isolated from carriage and disease, also indicated by the low number of repertoires appearing in both groups of isolates.

Different Opa proteins exhibit binding specificities for a range of human receptors including members of the CEACAM family (28, 43). The data presented here are consistent with a view that particular combinations of Opa proteins confer different meningococcal genotypes with particular sets of binding specificities and that these specificities are stably associated with each genotype. In the case of disease-causing meningococci, these combinations and specificities may contribute to invasiveness, for example, particular Opa proteins may enhance colonisation and invasion of the host. Different variants may confer different effects on host immunity, since Opa proteins are known to elicit the release of proinflammatory cytokines from monocytes (21) and down-regulate the activation and proliferation of CD4+ T cells via CEACAM1 binding (2, 16). Currently, little is known about the function of more than a handful of Opa variants and it is difficult to infer the binding specificities of the variants identified here from the few that have been studied. By determining that different Opa variants are associated with carried clonal complexes compared to those causing invasive disease, the population data in this investigation may inform future studies aimed at understanding the functional role of different Opa variants in meningococcal pathogenesis.

An alternative explanation for the association between Opa repertoire and MLST genotype involves Opa antigenic diversity rather than function. The evolution and diversity of the meningococcal Opa repertoire is likely to be influenced by
diversifying selection pressure from host antibody responses. Indeed, Opa-specific antibodies have been detected after meningococcal carriage and after invasive disease (23, 38). Host population antibody responses against the meningococcal PorA protein, a porin located in the outer membrane, have been suggested to shape the diversity of the meningococcal population (11). We propose that host population antibody responses also effect the structuring of the Opa repertoire in a similar way, by selecting against meningococci sharing parts of their Opa repertoires with other, unrelated genotypes that may have been previously recognised by host immune defences. An immunological reason for the structuring of the diversity would not be mutually exclusive with a functional explanation, since the parts of Opa proteins containing epitopes for antibodies may be different from those conferring functional specificity. Furthermore, the structuring and evolution of the meningococcal Opa repertoire is highly likely to be influenced by the strength of both functional and immunological selective forces during carriage.

The association of particular Opa repertoires with hyperinvasive clonal complexes may explain also why these meningococci cause most invasive disease (6). Different Opa repertoires encode different receptor binding specificities (8, 28, 43), which are likely to influence both meningococcal invasiveness and the ability of the bacteria to regulate human immunity during carriage and disease. Consequently, it may be possible to target hyperinvasive meningococci using Opa protein-based vaccines, especially since Opa proteins elicit antibodies after immunisation with outer membrane vesicle vaccines and after meningococcal infection (25, 38). There are still important immunological questions surrounding this strategy however, such as the
effects of Opa proteins on arresting the proliferation of CD4+ T lymphocytes(16) and
whether these effects are clinically relevant during vaccination.

A trend was observed for an association between disease severity and the opaD locus
across the set of disease isolates, but was not statistically supported. The reason for
this potential link, which would require further investigation in a larger data set, is
unclear due to the number and diversity of Opa variants at this locus. No other
associations between opa alleles or Opa repertoires and severity were observed. The
factors affecting meningococcal clinical severity are incompletely understood and
previous investigations have explored the effects of genomic DNA load and lipo-
oligosaccharide (LOS) levels and their influence on host cytokine profiles(4, 12, 31).
Cytokine levels are critically important in influencing meningococcal disease severity.
In contrast to the association between LOS levels in the blood and clinical severity of
meningococcal disease, cytokine levels have been found to be identical between LOS-
expressing and LOS-negative bacteria, suggesting a LOS-independent pathway(4).
Given the biological activities mediated by Opa proteins, including promoting
invasion of host cells and stimulating cytokine release from monocytes(21), the lack
of an Opa effect on disease severity is interesting. One caveat of this is that although
we did not detect an association between clinical severity and particular Opa sequence
variants, differences in Opa expression levels among different clonal complexes,
rather than sequence diversity have yet to be fully explored and may be important in
pathogenesis and severity. For example, previous studies have found that expression
of Opa proteins upregulates the expression of CEACAM receptors on host cells(29),
which leads to increased adhesion of meningococci(3). Furthermore, while this study
has focussed on the diversity of Opa proteins, little is also known about genetic
variation in CEACAM, the major Opa receptor, and whether particular genotypes or haplotypes influence severity or susceptibility to invasive meningococcal disease, perhaps by allowing increased uptake of meningococci via Opa interaction.

In conclusion, the meningococcal Opa repertoire was found to be strongly linked to MLST genotypes, irrespective of epidemiological sampling. Since MLST genotype also identifies hyperinvasive meningococci, particular Opa repertoires correlate with disease. The Opa repertoire was not, however, strongly associated with the severity of meningococcal disease, suggesting that other host and meningococcal factors may be more important than variation in Opa in determining differences in the clinical severity of disease.
Acknowledgements

The authors wish to acknowledge the aid of staff at St. Mary’s Hospital London in collecting human blood samples, purifying genomic DNA and for assembling clinical severity scores. The assistance of Roisin Ure for DNA extraction and the departmental DNA sequencing service at the University of Oxford Department of Zoology for their aid in separation of nucleotide sequence reactions are also acknowledged. This study was funded by the Meningitis Research Foundation (MRF), Bristol UK, as part of project 02/02. AJP and MCJM are named as inventors and MJC as a contributor on patent applications in the area of serogroup B meningococcal vaccine development. No other authors report conflicts of interest.
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Figure and table legends

Figure 1. Schematic of an opa gene, including the variable regions sequenced in this study and the relationship of these sequences to the predicted secondary Opa protein structure (reprinted from Callaghan et al. Infect Immun. 2006 Sep;74(9):5085-94).

Figure 2: Comparison of HVRC frequency in meningococci from invasive disease (black bars) vs. asymptomatic carriage (grey bars). Only HVRCs above 2% frequency are shown (see supplementary table 1 for full data set).

Figure 3: Multidimensional scaling (MDS) scatter plot showing distances between Opa repertoire of carriage and disease isolates in three dimensions of sequence space. Isolates have then been coloured according to clonal complex to indicate associations between antigenic and genotypic repertoires.

Figure 4: Multidimensional scaling (MDS) scatter plot showing distances between Opa repertoires of carriage and disease isolates in three dimensions of sequence space. Isolates have been coloured according to carriage or disease status.

Table 1. Diversity of Opa proteins in isolates from invasive disease. The Opa diversity is determined by alleles, variable region sequences (SV Vars., HV1 Vars. and HV2 Vars. indicate the numbers of sequence variants observed), sequence families (indicated by ‘Fam.’) and HV1-HV2 sequence family combinations (HVRCs). Percentage distances are given for nucleotide alleles and amino acid sequence data.
Table 2. Diversity of Opa proteins in isolates from asymptomatic carriage. The Opa diversity is determined by alleles, variable region sequences (SV Vars., HV1 Vars. and HV2 Vars. indicate the numbers of sequence variants observed), sequence families (indicated by ‘Fam.’) and HV1-HV2 sequence family combinations (HVRCs). Percentage distances are given for nucleotide alleles and amino acid sequence data.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
### Table 1.

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<th>SV Vars.</th>
<th>SV Fam.</th>
<th>HV1 Vars.</th>
<th>HV1 Fam.</th>
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### Table 2.

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