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„The impact of the expression of MAOA gene and HLA polymorphism in human pair bonding“

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1. Abstract

Monoamine oxidase A (*MAOA*) is an enzyme capable of catalyze the oxidative deamination of serotonin, noradrenalin and dopamine. All three amines are neurotransmitters and play important roles in signal transmission in neurons. The gene is coded on the X chromosome in humans, therefore females have two copies and males only one. Studies suggest that the amount of expression is associated with social behavior and aggression. During brain development, low expression levels of the gene can also lead to various diseases (for example Brunner syndrome).

A method to test the expression level of *MAOA* in a living organism is to genotype the gene. In the promoter region of *MAOA* a variable number of tandem repeats (MAOA-uVNTR) act as a regulative genetic element and influence the amount of the expression of the gene (Sabol et al., 1998). In the current literature the VNTR is described in form of 2, 3, 3.5, 4 or 5 repeats of a 30 nucleotide long sequence. The *MAOA* gene associated with 2, 3 or 5 VNTR repeats show lower levels of gene expression (*MAOA-L*) than the *MAOA* gene with 3.5 and 4 repeats (*MAOA-H*) (Sabol et al., 1998). If the expression level of *MAOA* is low (*MAOA-L*), an antisocial and aggressive behavior can be observed (Buckholtz et al., 2008). However, so far no studies have investigated the impact of MAOA-uVNTR polymorphism on pair bonding in humans.

We hypothesise these differences in *MAOA* gene expression levels may influence pair bounding in humans. Furthermore, previous studies have reported, that mate choice in vertebrates, including humans, is influenced by genes of the major histocompatibility complex (MHC; in humans also called human leukocyte antigens (HLA)). The highly polymorphic MHC genes express molecules that present antigenic peptides on the cell surface to T-cells, thereby initiating the T-cell mediated immune responses. Studies suggest that heterozygosity at MHC loci enhance resistance to infectious diseases by binding and presenting a wider range of antigens to T-cells (i.e., heterozygosity advantage). Moreover, sexual selection theory suggests that female choice partners carrying dissimilar genes at the MHC may play a role in increasing and maintaining genetic variation at these loci, thereby improving the

immunity of the offspring. Thus, we hypothesise that human females will choose male partners with dissimilar MHC alleles (MHC-disassortative mate choice) to increase genetic diversity of their offspring and avoid inbreeding. On the other hand, we expect that females will choose males with similar *MAOA* variants (*MAOA*-assortative mate choice) because of the social compatibility between the partners.

Our study indeed shows a significant difference between the dissimilarity of the HLA-B genes in married couples compared to the dissimilarity in random individuals (MHC-disassortative mate choice). This supports our hypothesis of disassortative mating at the MHC gene complex in humans. However, no significant difference could be found on the other studied MHC genes (HLA-A, HLA-DR1). We could also not find any significant association between the MAOA-uVNTRs and partner choice in our study of married couples, suggesting there is no influence of the *MAOA* gene variation on mate choice (*MAOA*-assortative mate choice).

2. Zusammenfassung

Monoaminooxidase A (*MAOA*) ist ein Enzym, welches die oxidative Desaminierung von Serotonin, Noradrenalin und Dopamin katalysiert. Die drei Amine sind Neurotransmitter und spielen eine wichtige Rolle bei der Signalübertragung in Nervenzellen. Das Gen ist beim Menschen auf dem X-Chromosom kodiert, was bedeutet, dass Frauen zwei, Männer jedoch nur eine Kopie besitzen. Eine niedrige Expression des Gens während der Gehirnentwicklung kann zu verschiedenen Krankheiten (z.b.: Brunner Syndrom) führen. Die Menge der Expression steht auch in direkter Verbindung mit dem sozialen Verhalten. Wenn das Expressionsniveau von *MAOA* niedrig ist (*MAOA-L*), kann ein antisoziales und aggressives Verhalten beobachtet werden (Buckholtz et al., 2008).

Eine Methode, um das Expressionslevel von *MAOA* in einem lebenden Organismus zu testen, ist das genotypen des Gens. In der Promotorregion von *MAOA* befindet sich eine Minisatelliten-DNA (MAOA-uVNTR), welche direkten Einfluss auf die Expression des Gens hat (Sabol et al., 1998). Der VNTR kann aus 2, 3, 3.5, 4 oder 5 Wiederholungen einer 30 Nukleotide lange Sequenz bestehen. Das Gen mit 2, 3 oder 5 Wiederholungen im VNTR wird auf einem niedrigeren Niveau exprimiert (Sabol et al., 1998).

Meine Hypothese ist, dass diese Unterschiede im Expressionsniveau die Partnerwahl beim Menschen teilweise beeinflusst. Des weiteren haben Studien bestätigt, dass die Partnerwahl bei Wirbeltieren, inklusive Menschen, von Genen des Haupthistokompatibilitätskomplex (MHC) beeinflusst wird. Die hoch polymorphen MHC Gene kodieren Moleküle welche Antigene an der Zelloberfläche den T-Zellen präsentieren und so die T-Tellen vermittelte Immunantwort aus lösen. Studien lassen vermuten, dass Heterozygosität am MHC Loci den Widerstand gegen Infektionskrankheiten erhöht, da eine größere Anzahl an verschiedenen Antigenen gebunden und präsentiert werden kann (heterozygosity advantage). Des weiteren deuten Studien zur sexuellen Selektion darauf hin, dass die Wahl von Sexualpartnern mit ungleichem MHC eine Rolle bei der Instandhaltung und Erhöhung der genetischen Vielfalt am MHC Loci spielt und so die Immunität der Nachkommen erhöht. Wir vermuten deswegen, dass Frauen Männer mit ungleichen

MHC Allele als Partner wählen (MHC-disassortative Partnerwahl) um die genetische Diversität ihrer Nachkommen zu erhöhen und um Inzucht zu vermeiden. In Hinblick auf das *MAOA* Gen vermuten wir, dass Frauen Männer mit ähnlichen *MAOA* Varianten wählen (MAOA-assortative Partnerwahl), um die Wahrscheinlichkeit der sozialen Kompatiblität zwischen den Partnern zu erhöhen.

In unserer Studie konnten wir einen signifikanten Unterschied zwischen der HLA-B Genen Ungleichheit in verheirateten Paaren im Vergleich zur Ungleichheit in zufällig ausgewählten Individuen finden (MHC-disassortative Partnerwahl). Diese Ergebnisse unterstützten unsere Hypothese der disassortativen Partnerwahl am MHC Gen Komplex. Allerdings konnten wir keinen signifikanten Unterschied am HLA-A oder HLA-DR1 Gen finden. Weiters konnten wir keinen signifikanten Zusammenhang zwischen dem MAOA-uVNTRs und der Partnerwahl in den getesteten verheirateten Paaren finden. Daraus kann schlussgefolgert werden, dass MAOA Genvariationen keinen Einfluss auf die Partnerwahl haben (MAOA assortative Partnerwahl).

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3. List of Abbreviations

ARMS	Amplification-refractory mutation system
CLIP	Class II-associated Ii peptide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphates
EDAR	Ectodysplasin A receptor
EN	Epinephrine
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FAD	Flavin adenine dinucleotide
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HWE	Hardy–Weinberg equilibrium
Ii	Invariant chain
LAO	L-amino acid oxidases
LD	Linkage disequilibrium
MAOA	Monoamine oxidase A
MAOB	Monoamine oxidase B
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
NE	Norepinephrine
PAO	Polyamine oxidase
PCR	Polymerase chain reaction
PEA	Phenylethylamine
PET	Positron emission tomography

List of Abbrevations

PLC	Peptide loading complex
qPCR	Real Time Quantitative PCR
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SSP	Sequence specific primer
TAP	Transporter associated with antigen presentation
TCP	T-cell receptors
VNTR	Variable number of tandem repeats
XIC	X-chromosome inactivation

4. Introduction

Sexual selection is a mode of selection where individuals of the same species and sex compete for access to the other sex to mate with and where one sex chooses mates of the other sex (Hosken and House, 2011; Darwin, 1859). This sort of selection is thought to be stronger than the natural selection (Hosken and House, 2011). In most mammals females choose the male partner (female choice) and the male compete with each other. The female individual chooses a male mate depending on his phenotypic traits indicating their genetic quality (Petrie, 1994). An example for gene associated mate choice is the major histocompatibility complex (MHC) complex and its influence on mate preference. The MHC encompasses several genes that are encoding cell surface proteins essential for the adaptive immune system. Several studies suggest that females choose partners with different MHC alleles in order to increase the level of heterozygosity and therefore the fitness of the offspring (heterozygosity advantage) (Havlicek & Roberts, 2009; Laurent, R. and Chaix, R., 2012; Carrington et al., 1999; Penn, 2002; Wedekind et al., 1997). Furthermore, in respect to humans, an important factor in human mate choice could be a matching behavior of partners because of the fact that human children need substantial paternal care (Marlowe, 2000). The behavior is to some extent also determined by genes and their expression level (Buckholtz et al., 2008). As shown in some studies (McDermotta et al., 2008; Fergusson et al., 2012) the *MAOA* gene and its expression level potentially influences the social behavior and level of aggression in humans and may therefore also play an important role in human mate choice.

4.1 Monoamine oxidase A

Monoamine oxidase was originally discovered by Mary Bernheim and called tyramine oxidase (Hare ML, 1928). There are two isozymes of monoamine oxidase in mammals, monoamine oxidase A (*MAOA*) and monoamine oxidase B (*MAOB*). Both enzymes contain a flavin adenine dinucleotide (FAD) bound to a cysteine residue (Son et al., 2008). They can be found expressed in neurons and astroglia. *MAOA* is also present in liver, pulmonary vascular endothelium, gastrointestinal tract, placenta, and in mitochondria of nearly every tissue cells (https://gtexportal.org/home). In the brain, it is localized in the outer mitochondrial membrane in the presynaptic terminal

of monoamine projection neurons to degrade serotonin presynaptically and expressed by neuroglia to degrade serotonin extrasynaptically (Westlund et al., 1988; 1993). *MAOB* is present in the mitochondria and nucleoli of platelets (https://gtexportal.org/home). The two enzymes are encoded by two linked genes, arranged on the short arm of the chromosome Xp11.23 and comprise 15 exons. The monoamine oxidase belongs to the same enzyme family of amino oxidases as maize polyamine oxidase (*PAO*) and L-amino acid oxidases (*LAO*). Both enzymes catalyze the degradation of several different biological amines (Figure 1). *MAOB* is irreversible inhibited by deprenyl and catalyzes the oxidation of phenylethylamines (PEA) and benzyl amine. *MAOA* is irreversible inhibited by clorgyline and catalyzes the oxidation of neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE) and epinephrine (EN) (Gaweska et al., 2011, Wang et al., 2012).

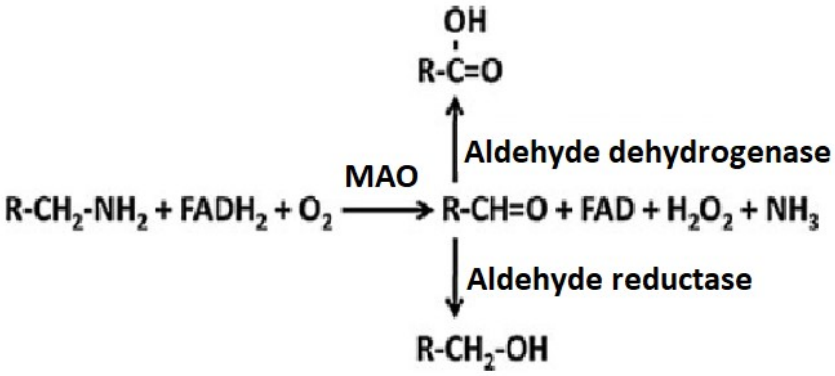

Figure 1: Oxidative deamination of monoamines by MAO with further metabolization by Aldehyde dehydrogenase and Aldehyde reductase. Adapted from Wang et al., 2012

MAOA contains a flavin binding domain, a substrate binding domain and a membrane binding domain (Figure 2) and is an important regulator of neurotransmitter signaling in the human brain. The human MAOA crystalize as a monomer while human MAOB crystalize as a dimer (Edmondson et al., 2007). The active side of *MAOA* has a monopartite cavity formed by a 6-residue loop (residues 210 – 216 in human *MAOA*). The conformation of this loop affects the shape and size of the binding substrate and therefore contributes to the specificity of the enzyme. The expression of the enzyme in humans is regulated by the transcription factors Sp1

(specificity protein 1), GATA2 (GATA binding protein) and TBP (TATA binding protein) (Gupta et al., 2015).

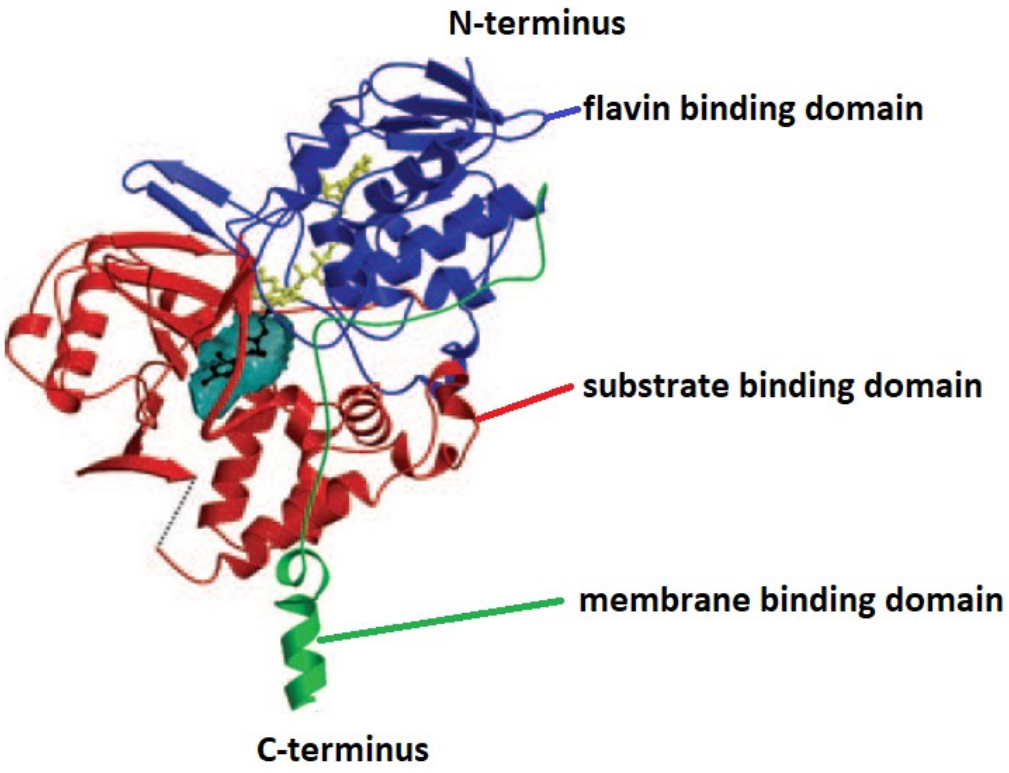

Figure 2: 3D structure of *MAOA* showing the flavin, substrate and membrane binding domains. Adapted from Edmondson et al., 2006

Expression dynamics, neuronal localization patterns and substrate preference characteristics strongly suggest that *MAOA* is a key factor in regulating the clearance and release of serotonin and norepinephrine during brain development and affects the behavior of the adult individual (Buckholtz et al., 2008). Studies linked diminished levels of serotonin to antisocial and impulsive behavior (Passamonti et al., 2005). An increasing level of amine in neurons is also linked to depression, senility, Alzheimer's disease, Parkinson's disease and schizophrenia (Silverm et al., 1995, Zhou and N. Panchuk-Voloshina, 1997; Zhou et al., 2006; Park et al., 2013). Due to the fact that the *MAOA* is X linked (coded on the X chromosome), only women can be heterozygote. Surprisingly, the influence of the MAOA-uVNTR phenotype on the brain structure and function in females is not as strong as in males (Buckholtz et al, 2008). Psychiatric syndromes like borderline and antisocial personality disorder are

also associated with the expression level of *MAOA* (Caspi et al., 2002, Fergusson et al., 2012).

4.1.1 Monoamine oxidase A-uVNTR

The promoter region of *MAOA*, approximately 1 to 1.2 kb upstream of the ATG translation initiation codon, contains a 30 base pair VNTR (MAOA-uVNTR). So far five different VNTR polymorphisms with different numbers of repeats in the promoter region have been determined: *MAOA*-uVNTR polymorphism with 2, 3, 3.5, 4 and 5 repeats (Figure 3). The 3 and 4 repeats are the most common alleles in human populations (Guo et al., 2008). The VNTR has a regulative function in gene expression due to its position in the promoter region. The VNTR alleles with 3,5 or 4 repeats seems to have the optimal length and act as an upstream activator, leading to the expression of high amounts of *MAOA* (*MAOA-H*) (Sabol et al., 1998, Balciuniene et al., 2002; Zhang et al., 2010), alleles with 2, 3 or 5 repeats causing low expression of *MAOA* (*MAOA-L*). However, it is not known if the VNTR influences the transcription of *MAOA* by serving as a binding site for an activator or by altering the chromatin structure (Passamonti et al, 2005).

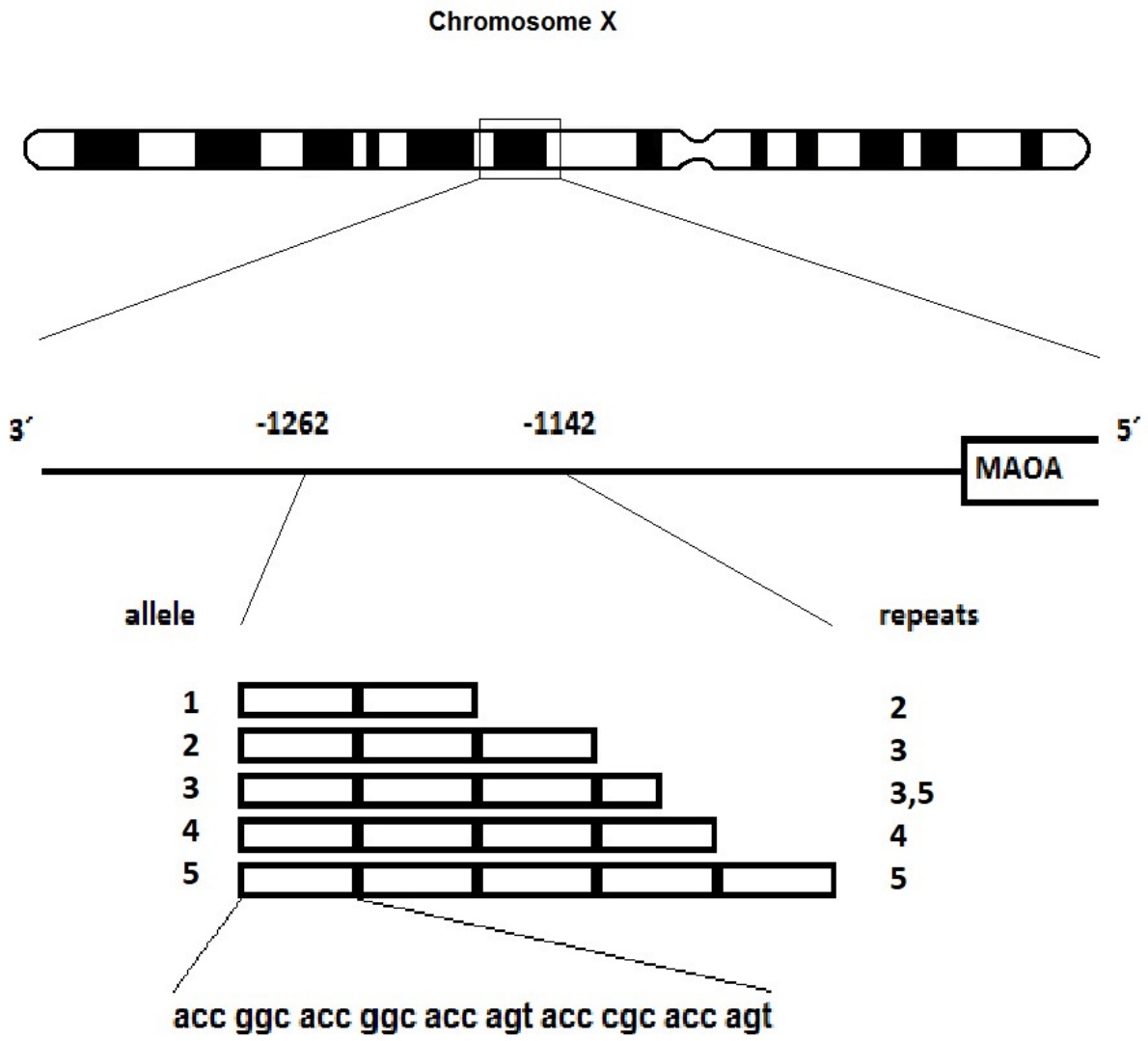

Figure 3: Location on chromosome X and structure of the Monoamine oxidase A-uVNTR in the promoter region of *MAOA* and the 30 base pair long repeats.

4.1.3 Aggression depending on the level of expression of MAOA

As already mentioned before, the expression level of *MAOA* strongly depends on the number of repeats in a VNTR in its promoter region, the MAOA-uVNTR (Sabol et al, 1998). However, the 2R allele was not examined in this study. Clinical work indicates the important role of *MAOA* in aggressive, impulsive and antisocial behavior (Passamonti et al., 2005; Choe et al., 2014; Ficks et al., 2014). The impulsive behavior is characterized by reduced information processing and the intolerance of delayed rewarding (Stein et al., 1995). Genetic deletion studies in animals (*MAOA* knockout in mice) showed alterations in the brain structure and function in

dependence of serotonergic and noradrenergic metabolism during the development (Shih et al., 1999). Observed male mice also showed hyperaggressive behavior and an enhanced fear response (Cases et al., 1995). Retardation and aggressive behavior was also found among eight men in a Dutch family with a point mutation in the *MAOA* gene leading to an enzymatic deficiency of *MAOA* (Brunner et al., 1993). The fact that *MAOA* expression is linked to these behaviors suggests its important role in brain development. In male humans with the *MAOA-L* genotype the aggression and antisocial behavior is much more pronounced compared to humans with the *MAOA-H* genotype. The reason for the higher amount of aggression is either that humans with the *MAOA-L* genotype are hyposensitive and care less about harming others or that they are hypersensitive and overreact more easily (McDermott et al., 2008). Biomarker for impulsivity and aggression where also used for proving this theory (Buckholtz et al, 2008). There is also a decrease in gray matter volume associated with the *MAOA-L* genotype of about 8% and an increase in lateral orbitofrontal volume of 11%. The increased lateral orbitofrontal volume (relevant in emotion and rewards in decision making) was only found in males (Buckholtz et al., 2008). An association has been detected for the *MAOA-L* genotype and antisocial and aggressive behavior in males with an abusive childhood (Buckholtz et al., 2008). In that case, *MAOA-L* had a negative effect on the brain development. Studies showed that the *MAOA* genotype, sex and early-life experience are important factors for antisocial behavior later in life (Caspi et al., 2002). *MAOA-L* males are more vulnerable to negative early life experiences, where *MAOA-H* males are less susceptible to it (Table 1).

Table 1: Association of *MAOA* genotype, early life experience, and the resulting behavior as an adult. Adapted from Buckholtz et al., 2008

Maoa genotype	early life experience	behavior as an adult
MAOA-H	positive	normal behavior
MAOA-H	abusive	normal behavior
MAOA-L	positive	normal behavior
MAOA-L	abusive	antisocial behavior

Studies of *MAOA* genetic showed a greater vulnerability of men to the effects of the *MAOA-L* allele on brain structure and behavior (Buckholtz et al., 2008). It is unlikely that a gene dosage effect of the X-linked *MAOA* gene can explain the gender difference in behavior (Moffitt et al., 2001) because in females only one *MAOA* allele is expressed in a single cell duo to X-chromosome inactivation (XIC) (i.e., one of the two X-chromosomes is inactivated in each cell). Female *MAOA-L* homozygotes and heterozygotes have the same neural response as hemizygous males (Buckholtz et al., 2008). However, newer studies showed a three way interaction between sex, *MAOA* genotype, and amygdala activity in regards to reactive aggression (Holz et al., 2014). In contrast to MAOA-H males, males carrying the *MAOA-L* display an increased activity in the amygdala and hippocampus under stress. In females, the *MAOA* genotype has the opposite effect on the activity of the amygdala and hippocampus (Holz et al., 2014). However, the reasons for different effects on males and females are most likely sex-specific hormones. The activity of *MAOA* is directly regulated by estrogen. Estrogen receptors are expressed in amygdala and orbitofrontal cortex (MacLusky et al., 1986) where they regulate the transcription of *MAOA* (Gundlah et al., 2002). The *MAOA-L* allele has little to no effect on female brain development and behavior (Chakravorty and Halbreich, 1997; MacLusky et al., 1986; Gundlah et al., 2002; Ou et al., 2006). In males, testosterone may influence the transcription of *MAOA* by acting through glucocorticoid/androgen response elements in the *MAOA* promoter (Ou et al., 2006). Another study showed that the *MAOA* genotype mainly influences the behavior of males and not the behavior of females because of the fact that the epigenetic modification of the *MAOA* gene influencing its expression plays an important role in woman, but apparently not in men (Wong et al., 2010) An interesting question is why the *MAOA-L* allele is still maintained in the population even though it promotes antisocial behavior. A theory is, if everyone would have *MAOA-H*, there would be a niche to exploit for *MAOA-L*. If everyone would have *MAOA-L*, the positive effect would disappear. In this case, this theory seems to be true indicating that negative frequency-dependent selection acts as primary evolutionary mechanism in maintaining the MAOA-uVNTR in human populations.

4.1.4 Different distribution of expression level of MAOA in human population

Different human populations have different distributions of *MAOA-L* and *MAOA-H* alleles. In the Caucasian population, 34% carry the *MAOA* allele with 3R (*MAOA-L*). In this population, the 3R and 4R are the most common alleles. In the Maori population, approximately 56% carry the allele with 3R (*MAOA-L*). The allele with 3R is also found in, 77% of Chinese males and in 59% of African males (Sabol et al., 1998; Lea and Chambers, 2007). The 2R allele (*MAOA-L*) occurs only in 5.5% of the African-American population and in 0.1% of the Caucasian population (Beaver et al., 2012).

4.2 MHC Complex

The major histocompatibility complex (MHC) that is also called HLA (human leukocyte antigen) in humans, is a gene dense region with more than 240 genes that spans around 4Mbp on chromosome 6 (6p21.3) (Figure 4). The HLA system was first described by J. Dausset in 1965 who named it Hu-1. The complex was later known as HLA complex. The coded molecules are responsible for the antigen presentation on the cell surface.

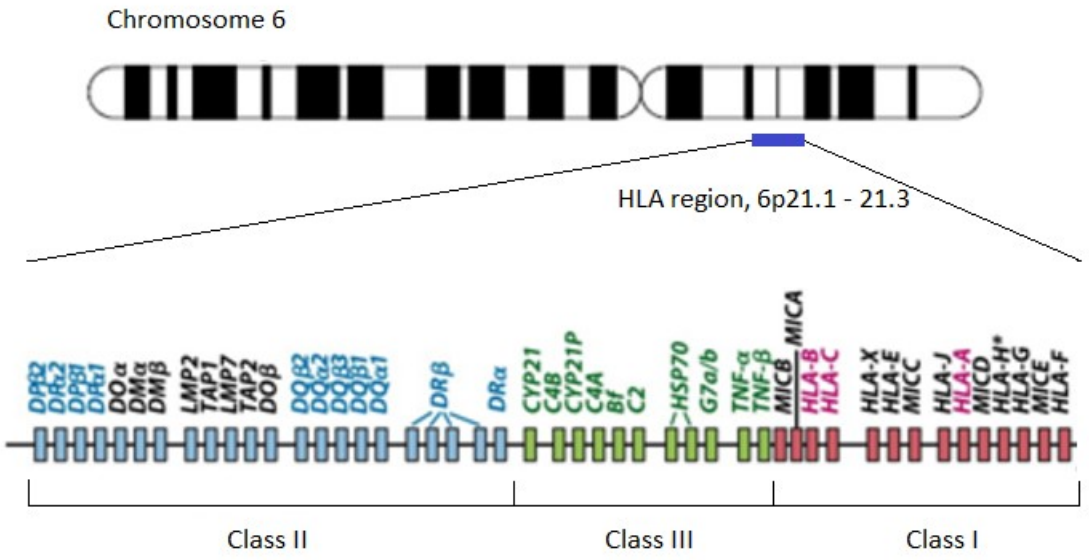

Figure 4: Chromosome 6 with the enlarged HLA region. The HLA region consists of MHC class I, MHC class II and MHC class III genes. Adapted from www.researchgate.net

The classical MHC consists of class I and class II genes. These highly polymorphic MHC genes express molecules on the cell surface binding and presenting antigenic peptides on the cell surface to different T-cells, thereby initiating the T-cell mediated immune responses. The genes in the MHC class I region are coding for the classic HLA class I HLA-A, HLA-B and HLA-C heavy chains and the non-classic HLA class I HLA-E, HLA-F, HLA-G, MICA and MICB proteins. All these proteins with the exception of MICA and MICB forming heterodimers with the β2-microclobulin chain coded by a gene on chromosome 15 (Goldberg et al., 2015). MHC class I molecules are expressed by all nucleated cells and presenting protein fragments with origins in the cytosol and the nucleus on the cell surface to CD8+ T-cells. In the MHC-class 1 pathway antigens are degraded by proteasomes to 8 – 9 amino acid long fragments. These fragments are than translocated to the endoplasmic reticulum (ER) via transporter associated with antigen presentation (TAP). In the ER the MHC class I heterodimer is assembled by the polymorphic heavy chain (α) and the β2-microglobulin chain (Neefjes et al., 2011) (Figure 5).

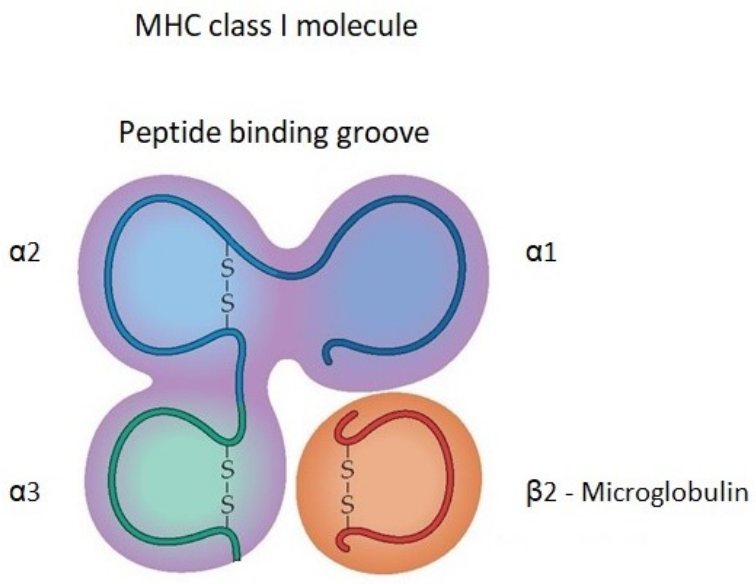

Figure 5. Fully assembled MHC class I molecule consisting of a heavy chain α and a β2-microglubin chain. Adapted from http://what-when-how.com/acp-

medicine/adaptive-immunity-histocompatibility-antigens-and-immune-response-genes-part-1/

The peptide loading complex (PLC) is in charge of loading a peptide into the MHC class I peptide binding groove and stabilize the complex (Basu et al., 1999; Spee et al., 1997). The fully assembled peptide-MHC class I complex leaves the ER and is transported via the Golgi to the plasma membrane for antigen presentation (Figure 6). MHC class I molecules which are not capable to bind a peptide are degenerated by the ER-associated protein degradation (ERAD) system (Hughes et al., 1997). On the cell surface, the peptide-MHC class I complex is recognized by the T-cell receptors (TCP) expressed on the surface of CD8+ T-cells. Activated CD8+ T-cells (T cytotoxic cells) will than destroy the infected host cells (MHC class I pathway) (Blum et al., 2013). This rout of antigen presentation is primary suited for the presentation of viral peptides from infected cells (Goldberg et al., 2015).

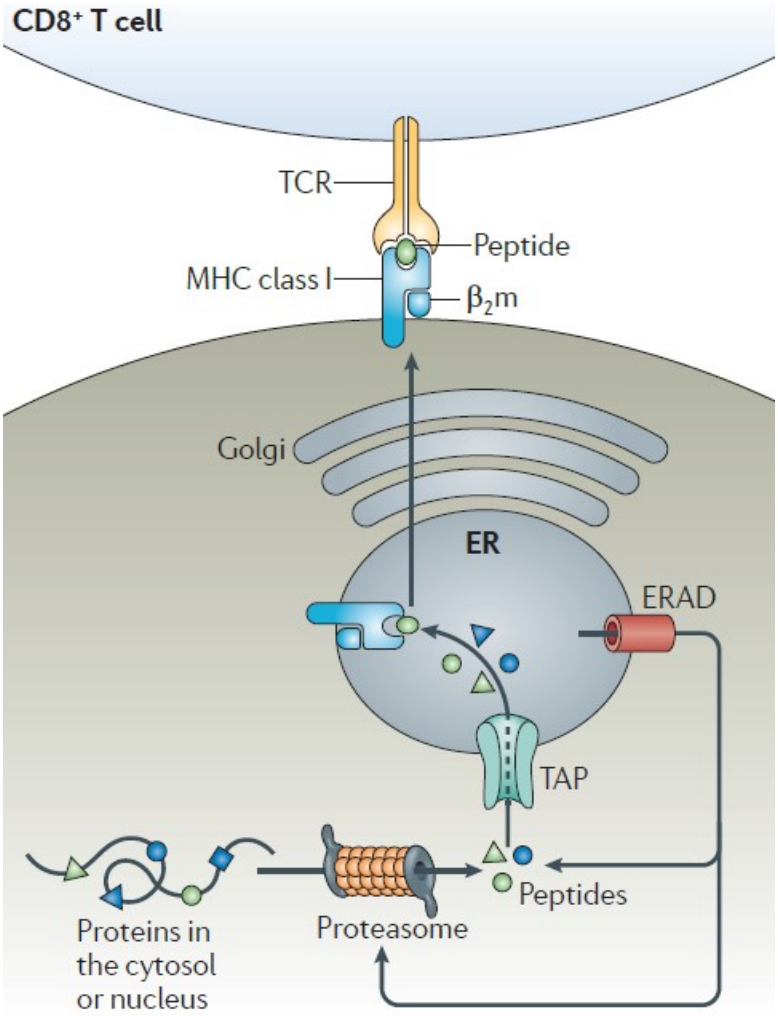

Figure 6. MHC class I pathway. At first antigens are degraded by the proteasome and transported into the ER via transporter associated with antigen presentation (TAP). In the ER the antigen is inserted into the binding groove of the MHC class I molecule by PLC. The peptide–MHC class I complex is transported via the Golgi to the cell surface for antigen presentation to CD8+ T-cells. MHC class I molecules which are unable to bind an antigen are degraded by ERAD. Adapted from Neefjes et al., 2011

Like the MHC class I heavy chain, the MHC class II heavy chain is also coded by polymorphic genes named HLA-DR, HLA-DQ, HLA-DP, HLA-DM and HLA-DO. Unlike MHC class I genes, the MHC class II genes are coding for both chains (α and β chain) to form the functional MHC class II heterodimer (Goldberg et al., 2015). MHC class II molecules are predominantly expressed on antigen presenting cells

(APC) like macrophages, dendritic cells, Langerhans cells, B lymphocytes and Kupffer cells. Under certain circumstances thyrocytes and intestinal epithelial cells are also capable to express MHC class II genes (Wu et al., 1999, Hershberg et al., 1998). MHC class II molecules present proteins with their origin outside of the cell on the cell surface to CD4+ T-cells. Therefore the MHC class II molecule is assembled in the ER using one α chain and one β chain forming a heterodimer (Figure 7).

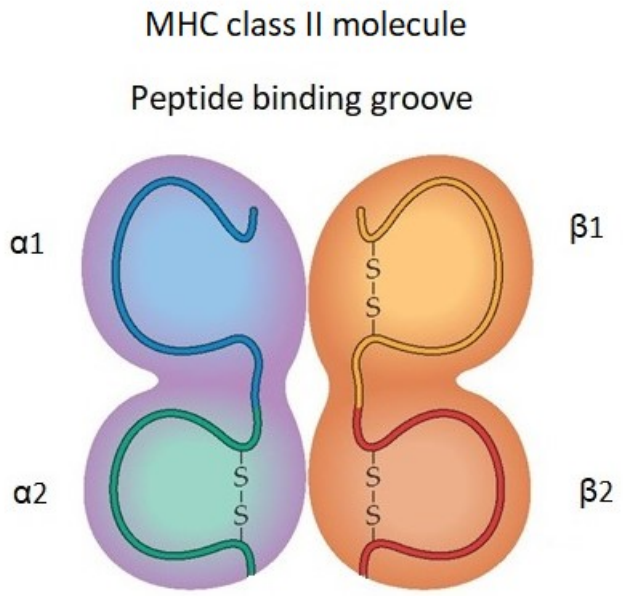

Figure 7. Fully assembled MHC class II molecule consisting of an α chain and a β chain. Adapted from http://what-when-how.com/acp-medicine/adaptive-immunity-histocompatibility-antigens-and-immune-response-genes-part-1/

An invariant chain (Ii) is than inserted into the binding groove and the Ii-MHC class II complex is transported to a late endosomal compartment named MHC class II compartment (MIIC). Peptides from a protein degraded in the endosomal pathway are also transported to the MIIC. The Ii protein bound to the MHC class II molecule gets digested and leaves a residual class II-associated Ii peptide (CLIP) in the peptide binding groove. This CLIP will then be exchanged for a specific peptide (Neefjes et al., 2011). Unlike the MHC class I molecule, the MHC class II molecule will bind peptides longer than its binding groove of 9 amino acids (Carson et al., 1997). Finally the peptide MHC class II complex is transported to the plasma membrane to present the antigens to CD4+ T-cells (Figure 8). The peptides presented by the MHC class II pathway mainly originate from extra cellular

pathogens. The activated CD4+ T-cells can activate B-cells which will produce antibodies to bind and neutralize the antigen (MHC class II pathway) (Blum et al., 2013). In this work we studied the HLA genes HLA-A, HLA-B and HLA-DRB. HLA-A and HLA-B belong to the MHC class I, the HLA-DRB belongs to MHC class II.

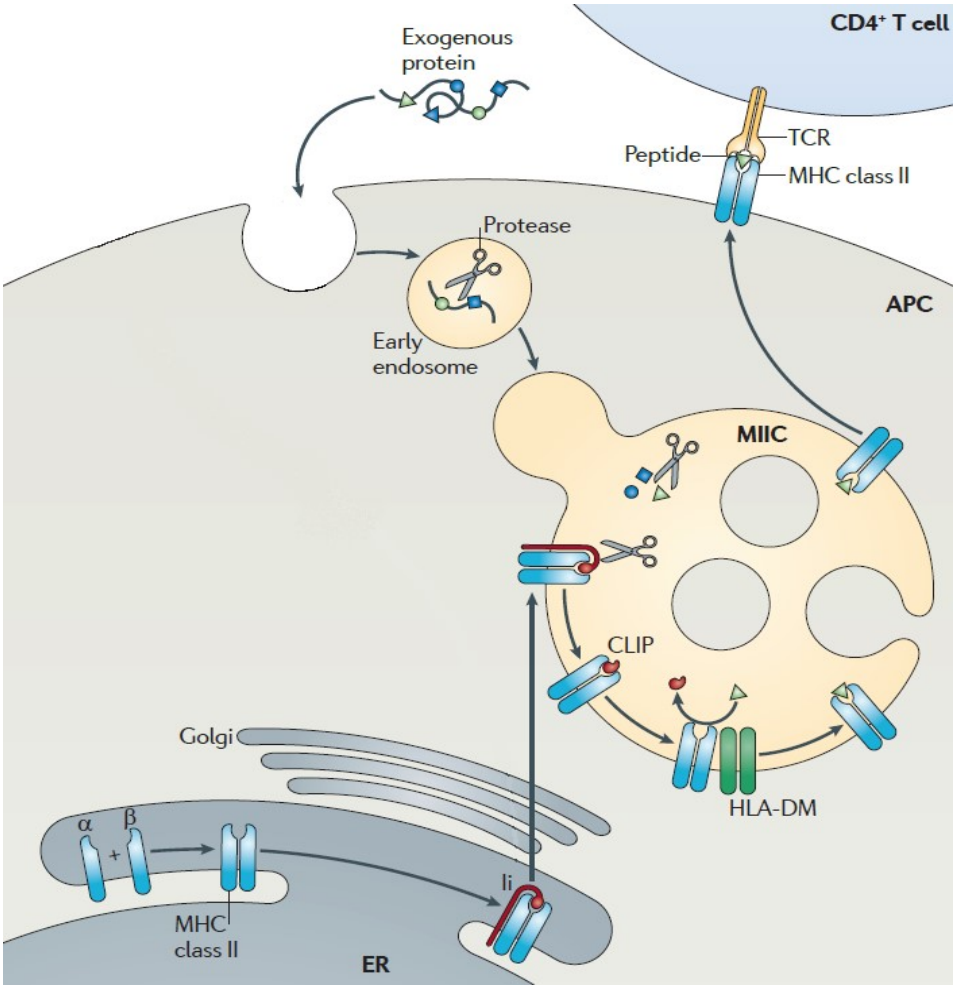

Figure 8. MHC class II pathway. The α and β chain are assembled in the ER to form the MHC class II molecule. The invariant chain (Ii) is than inserted in the binding groove of the MHC class II molecules. The Ii-MHC class II complex is transported through the Golgi to the MHC class II compartment (MIIC) where the Ii gets degraded by proteases. The class II associated Ii peptide (CLIP) fragment stays in the MHC class II binding groove until it gets replaced with a peptide with the help of the HLA-DM chaperon. The peptide was originally an exogenous protein which than got degraded in an early endosome by a protease and transported to the MIIC. The peptide-MHC class II complex is transported to the plasma membrane to present the bound peptide to CD 4+ T cells. Adapted from Neefjes et al., 2011

In humans MHC class I and class II loci are usually closely linked (in high LD) and organized in a gene dense cluster. The classical HLA genes are highly polymorphic. There are four different theories trying to explain the phenomena (Havlicek & Roberts, 2009). The first theory is, that the polymorphism is maintained because the MHC expression is co-dominant and thus, a heterozygote individual will express more type of functional MHC proteins than homozygote ones and will consequently be able to respond to a greater range of pathogens (heterozygosity advantage). This leads to an average higher fitness of individuals in a population with a great variety of MHC alleles (Doherty and Zinkernagel, 1975; Hughes and Nei, 1988). However, on an individual level, an optimized and not maximized level of heterozygosity provides the highest fitness. This is because a too high diversity in the MHC allele might lead to having diminishing returns on T-cell diversity (Nowak et al., 1992). The second theory iindicates that the MHC polymorphism is a result of an arms race between the adaptive immune system and pathogens (negative frequency dependent selection). This theory is also called the Red Queen hypothesis (Leigh et al., 1973) and is used as a model to explain the advantages of sexual reproduction (Bell, 1982). The third theory postulates that the MHC diversity is maintained over time due to a varying degree of pathogen presence to the immune system (Hedrick, 2002). This is also called the fluctuating selection hypothesis. The diversity at the MHC is maintained by the spatial and temporal heterogeneity in the pathogens abundance (Hill et al., 1991). The fourth hypothesis is that the polymorphism is maintained by sexual selection. Individuals of one or both sexes prefer partners with dissimilar MHC genotype to their own (Jordan and Bruford, 1998; Penn, 2002). Duo to the benefits of a heterozygote HLA allele most vertebrates like fishes, mice, birds, primates and also humans have developed mechanism to identify the HLA genes of a potential partner. Immune genes of the MHC correlate with the body odour (Wedekind et al., 1995) and studies showed HLA-related odour preferences in human mate choice (Milinski et al., 2001). Women preferred the odour of shirts worn by men with different MHC alleles over men with more matching MHC alleles (Havlicek and Roberts, 2009; Herz and Inzlicht, 2002). There are also studies about the genetic relatedness of couples with mixed results, suggesting that the MHC genotype influences the mate choice in some but not all populations (Khankhanian et al., 2010; Ober, 1999; Derti et al., 2010). Therefore the body odour is important for sexual communication and to find a partner

with a compatible immune system (Hämmerli et al., 2012). Even though there are a lot of studies about the association between MHC alleles and human mate preferences, the role of HLA in human mate choice is still highly controversial.

4.3 The evolution of sex and sexual selection

Sexual reproduction requires two parents to combine their genetic material using gametes (haploid cells) to form a zygote. Gametes are formed by the parents after the genome undergoes a recombination during meiosis (Kleckner 1996). Although almost all higher animals and plants are reproducing through sex (only 0.1% of all animal species reproducing asexually (Vrijenhoek 1998)), the higher cost compared to asexual reproduction of a population with only females is difficult to explain. In particular the twofold cost of males compared to a population with asexual females is hard to explain (Hartfield et al., 2012). There are several theories addressing this problem and trying to explain the advantage of sexual reproduction. One direct advantage of sex (accounting for the evolution of sex due to an immediate effect) is that sexual reproduction repairs damaged DNA and regenerates the genome (Bernstein et al., 1988). Sexual selection may also function as basis, in particular in respect to immune defense genes, to generate a “moving target” against rapidly pathogens and parasites (Red Queen hypothesis, Leigh et al., 1973; Jaenike, 1978; Hamilton, 1980; Bell, 1982; Hamilton et al., 1990). Because this direct and short-term advantages fail to explain the evolution of sex completely, indirect population genetics-based hypotheses may also explain the advantages (Hartfield et al., 2012). These hypotheses predict an advantage of sexual reproduction due to the creation of sexually fitter individuals. These individuals also have genotypes with higher fitness which would not be possible by asexual reproduction (Williams et al., 1975). The combination of all of these theories might be the best explanation for overcoming the twofold cost of sexual reproduction (West et al., 1999). Sexual reproduction led to sexual selection in addition to natural selection. Sexual selection can be seen as a competition amongst individuals within a species for potential mating partners (Hosken and House, 2011). The mechanisms of sexual selection are mate choice and competition for mates (Darwin, 1859). Traits which are providing an advantage in securing a mate are driven by sexual selection whereas traits increasing the mean

fitness of a population are driven by natural selection (Hosken and House, 2011). A trait is subject to sexual selection when there is a link between the trait and mating success and reproductive success. This sexual selection for such traits is often more powerful than natural selection (Hosken and House, 2011). Usually the females choose the males and the males compete with each other. The initial evolution of female mate choice is the female´s preference for males with certain traits that gives them a reproductive advantage. However, this advantage can drive the trait values beyond the naturally selected optima (Fisher, 1958). This can happen because an increase in the male trait can lead to a small loss of fitness, but a large gain in mating advantage (O'Donald, 1977, 1980). The benefits gained by female choice can be divided in direct and indirect benefits. Direct benefits for females are increased lifespan and fecundity, indirect benefits are an increased quality of offspring, like more attractive sons (Fisher's effect) or general viability (good genes) (Hosken and House, 2011).

4.3.1 The link between mate choice and genes

To find a matching partner, members of one sex choose individuals of the other sex to mate with. Members of the same sex compete with each other over access to members of the other sex. This behavior is the basis for intersexual selection (Darwin et al., 1858; Starr et al., 2013). Studies in a wide variety of taxa showed that females apparently are able to examine the phenotypic quality traits of males which should also indicate their genetic quality (Petrie, 1994). A number of studies has focused on "good genes" indicators in human mate preference such as body shape (may indicates physical strength) (Fan et al., 2005) and size (Nettle, 2002). These preferences may either lead to indirect benefits like offspring variability or to direct benefits like paternal care and enhanced resource acquisition (Roberts and Little, 2008). Another good example for gene associated mate choice is the MHC complex, which is coding for cell surface proteins essential for the adaptive immune system. Most studies showed that choosing a partner with a different MHC complex (MHC disassortative mating) is beneficial because it will increase the level of heterozygosity of the offspring (Havlicek & Roberts, 2009). However odour preferences studies didn't answer the question if females prefer MHC dissimilar partners (Thornhill et al.,

2003; Wedekind and Fury, 1997; Wedekind et al., 1995) or MHC intermediately partner (Jacob et al., 2002). There are also some studies that did not show a significant association between MHC dissimilarity and partner choice (Chaix et al., 2008). Females not only choose partner in respect of the dissimilarity or similarity of the MHC genes, but also in respect of the MHC diversity of the partners (Lie et al., 2008, McClelland et al., 2003). MHC heterozygous individuals are presumably able to bind a wide range of different antigens and thus have better immune competence (heterozygosity advantage) and therefore the offspring should benefit from a longer period of high quality paternal care (Roberts et al., 2005b). The good-genes heterozygosity hypothesis (Brown, 1997, 1999) proposes that allele diversity should be preferred when it is a sign for individual quality. Therefore, a preference for MHC diversity could be an adaption because gaining access to high quality mates is beneficial. The observed disassortative mating pattern at the MHC in some human populations (Havlicek and Roberts, 2009) may be the result of preferring the same type by all individuals and has nothing to do with preferences of a like mate (Burley, 1983, Gimelfarb, 1988). Genes responsible for the social behavior of individuals may also play an important role in mate choice (Laurent et al., 2012). We hypothesise that choosing a partner with similar social behavior is advantageous to guarantee a better cooperation between the male and the female partner. Extreme patterns of kinship were found in genes among spouses which are involved in behavior (in the Yoruba in Ibadan, Nigeria (YRI) population) and regulation of behavior (in the Utah Residents with Northern and Western European Ancestry (CEU) population) (Laurent et al., 2012). Therefore, a matching partner should have similar alleles of genes affecting the social behavior. However, many factors affect the partner choice in humans. Human partner choice does not only depend on body odour preference, but also on social status and other culturally transmitted mating preferences. Such culturally transmitted mating preferences were observed by Laurent et al., 2012, in the Utah Residents with Northern and Western European Ancestry (CEU) population, where a significant kinship among spouses was found in genes associated with pigmentation. In the African Yorube (YRI) population, genes involved in growth and adult height showed a significant similarity in spouses. The influence of the MHC on mate choice is associated with many factors including the importance of social-cultural factors, the

level of the genetic diversity in the given population and the strength of the pathogenic pressure (Laurent and Chaix, 2012).
The monogamous mating system in most human populations has been attributed to the fact that human children need substantial paternal care (Lovejoy, 1981, Marlowe, 2000). This could be one of the main reasons, why it is important for human females to choose male partners with matching personality and behavior. Studies have shown that in some human cultures spouses were similar in their cooperativeness and generosity (Tognetti et al., 2014). This suggests that there exist a mate preference for more altruistic men. The preference for an altruistic partner could result from the cost involved (signal of mate quality) or because an altruistic behavior is a sign for parental investment and also important to survive in rural communities (Tognetti et al., 2014). Like other phenotypical traits, the behavior is to some extent also determined by genes and the expression level of the genes (Bergeman et al., 1990; Turner et al., 1995; Buckholtz et al., 2008). In this study, one of the main focuses was on one hand the *MAOA* gene which is known for affecting the social behavior in humans (McDermotta et al., 2008; Fergusson et al., 2012; Buckholtz et al., 2008) and on the other hand the monoamine oxidase A-uVNTR, a variable number of tandem repeats (VNTR) within the promoter region of *MAOA* influencing the gene expression level. There is also an association between the *MAOA* genes and the voluntary contribution in public good game (PGG) underlining the role of *MAOA* in social behavior (Mertins et al., 2011). Different genetic variants such as single nucleotide polymorphisms (SNPs) or VNTRs in the promoter region of a gene can modulate the gene expression. Two humans may have the same alleles of a gene, but they can show significant differences in the level of gene expression at this gene, leading potentially to different phenotypic expression. This will furthermore affect the behavior, aggressivity and personality of the person (Hyman, 2000). Therefore, it could be possible duo the impact of the *MAOA* gene on social behavior and aggresivity, that the MAOA-uVNTR (influencing the expression level of the gene) influences the female mate choice.

4.4 Does human mate choice depend on MAOA and MHC alleles

In view of the MHC allele most studies showed that individuals of one or both sexes choose partner with different MHC alleles to increase offspring heterozygosity (Carrington et al., 1999; Penn, 2002; Wedekind et al., 1997). This offspring will have better adaptive immune system than offspring from two partners with similar MHC alleles. On the other hand, some studies reported no association between the MHC and mate preference in humans (Khankhanian et al., 2010; Ober, 1999; Derti et al., 2010; Chaix et al., 2008). Thus, the role of MHC in human mate choice is still very controversial and most of the studies only tested for mate preferences and not for mate choice. For this reason we tested married couples of a homogenous group (couples from Switzerland all individuals with European genetic ancestry) to detect if females choose for pair-bonding partners with dissimilar MHC alleles (MHC-disassortative mate choice).

Unlike the MHC allele, *MAOA* does not affect the immune system but the aggression level and antisocial behavior of the male partner. Cooperative traits could be criteria for mating choice in humans. There are evidences that, under certain coditions, couples are similar regarding their contributions to a public good (Tognetti et al., 2014). As far as we know there are no studies published which analyzed the association between the *MAOA* genotype and mate choice in humans. For this reason, we tested for MAOA-assortative mate choice (females choose partners with similar MAOA alleles).

5. Materials and methods

5.1 Human samples and DNA extraction

Participants were recruited by basis note Ltd. from the larger Zurich area in Switzerland for a previous study. In total we analyzed 184 individuals with European genetic ancestry, consisting of 92 married couples. We studied the genetic variation of the human leukocyte antigens (HLA) and of the Monoamine oxidase A (*MAOA*) gene. Genomic DNA was extracted from human buccal swabs/saliva with the Gentra Puregene Buccal Cell Kit from QIAGEN protocol. Finally, the DNA was dissolved in hydration solution (1 mM EDTA, 10 mM Tris·Cl pH 7.5) and stored at –20°C in three 8x12 plates labeled HCE120808-1 (85 samples), HCE150908 (35 samples) and HCE061008 (64 samples). Samples with less than 40µL were filled with H_2O to reach at least 40µL of sample value. The DNA concentration in each sample was between 2 and 7ng/µL.

5.2 HLA typing

The Human Leukocyte Antigen (HLA; also called major histocompatibility complex (MHC)) genes are located on chromosome 6p21. In the present study the HLA class I genes, HLA-A, HLA-B and the HLA class II gene HLA-DRB1, were determined by sequence-specific primer (SSP) technology by the IMGM laboratories, Lochhamerstrasse, Martinsried, Germany. Multiple pairs of allele specific primers are used in this technology. The primers are cis-located to the gene of interest and were used to determine which allele is present in the tested DNA sample. The HLA genotype was determined only in 51 couples.

5.3 Monoamine oxidase A (MAOA): molecular typing

The *MAOA* gene is located on chromosome X (NC_000023.11, 43654907-43746824). The variable number tandem repeat (Monoamine oxidase A-uVNTR) was used to assess the genetic variability in our samples. In addition, we tested three single nucleotide polymorphisms (SNPs) for linkage disequilibrium (LD) with the

VNTR locus. The aim was to identify any SNPs that are in high LD with the VNTR that can be used subsequently as a marker for the determination of the allelic variation at the VNTR locus.

5.3.1 SNP typing

To genotype the different SNPs, a qPCR based method, using ARMS primer, was used. Three different primers for each SNP were designed. As a reference gene to compare the qPCR curves, the Homo c-MYC gene was used. In addition to the primers, probes were designed to bind the amplified DNA in the qPCR reaction. The probes were marked on the 5´end. Probes binding to the SNP amplicons were marked with FAM dye, the probe binding to the Homo c-MYC amplicon was marked with a HEX dye.

To design the ARMS primers for amplifying the SNPs we first downloaded the human DNA sequence around the SNPs of interest in fasta format from the NCBI database and saved the sequences as .doc files. Blanks and line breaks were removed. We used the following two different software programs to design specific primers: Perlprimer (http://perlprimer.sourceforge.net/, v1.1.21 for windows) and Primer Express 2.0 (Applied Biosystems, v2.0).

However, none of these programs provided any usable primers for this study. We concluded that the general structural complexity of the X-chromosome and the specific number of repeats at this genomic locus obstruct an efficient primer design by these software. Therefore, all primers were designed by hand.

To design the primer pair to amplify the human c-MYC gene, the gene sequence was also downloaded from the NCBI database in fasta format and saved in a .doc file. All blanks and line breaks were removed. For this gene the Primer Express 2.0 software program was used to design proper primers.

All designed primers were blasted against the whole human genome using the NCBI database and the megablast (highly similar sequences) nucleotide-nucleotide search algorithm provided on https://blast.ncbi.nlm.nih.gov to avoid sequence mismatches. For that reason, the primer sequences were copied in Primer Blast, choosing DNA as sequence type, “nr” as database and “human” as organism.

To calculate Tm for each primer pair, PrimerExpress 2.0 and OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) were used. When using the program

OligoAnalyzer 3.1, some settings used by the program by default were changed: the Mg^{++} concentration was changed to 1mM and the dNTPs concentration was changed to 0,2mM.
Mfold (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) was used to check the secondary structure of our expected amplicons and to calculate ΔG, the free energy in the system. A lower ΔG value implies a stronger secondary structure. To avoid secondary structures, only primers were used which would amplify amplicons with a ΔG value above -2kcal/mol. After entering the amplicon sequence, the ionic concentration of Na^{+} was changed to 50mM and the Mg^{++} concentration was changed to 2mM. The folding temperature was changed to 60°C.
Net Primer (Premier Biosoft, http://www.premierbiosoft.com/NetPrimer) was used to check interactions in between the primers. The temperature was changed to 60° C.
The designed primers and probes were ordered at Sigma-Aldrich Handles GmbH, Austria.
Following SNPs were observed: rs57411356, rs5953210, and rs7948702.

5.3.1.1 ARMS Primer

The ARMS primer method is a PCR based method which is used for detecting any mutation like deletions or single base changes (Ye et al., 2001). The abbreviation ARMS stands for amplification-refractory mutation system. The method is based on the principle of allele specific primers which are compliment with the target DNA sequence in the PCR process. The primers have one mismatch (called Taq-MAMA) on the second base counting from the 3`side (Li et al, 2004). This one mismatch would not be enough to prevent the primer to bind. Although, if there is a second mismatch caused by a mutation in the DNA the ARMS primer will not bind (Figure 9). Amplification is only possible, if there are no mismatches in the DNA other than the Taq-MAMA. To detect these mutations or SNPs, the qPCR curve from the ARMS primer is compared to the qPCR curve of a reference primer (Figure 10). Therefore three different primers for each SNP are needed: a SNP specific forward primer for one allele (one mismatch on the allele with the matching SNP), a SNP specific forward primer for the other allele (one mismatch on the allele with the matching SNP) and a reverse primer used for both alleles. The SNP specific primer for the wrong allele will not bind as good as the SNP specific primer for the right allele because of

the two mismatches. The result will be a delayed qPCR curve because the primer with only one mismatch will amplify their target much earlier (Figure 10). SNP specific ARMS primer and multiple qPCR reaction with different sets of primers, one set for each target SNP, were used for SNP genotyping (Figure 10). In our case, ARMS primer in combination with qPCR was used to find different SNPs in the promoter region of the human *MAOA* gene (Figure 11).

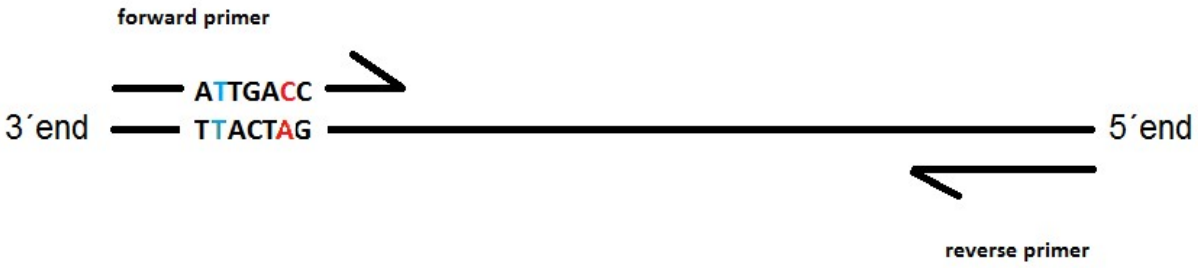

Figure 9. The nucleotide in the forward primer written in red is the intentional build in mismatch. The blue written nucleotide is the SNP we are trying to determine. In this example, the SNP is also a mismatch (an adenine nucleotide on the primer would be a positive match), thus the primer specificity will be very weak.

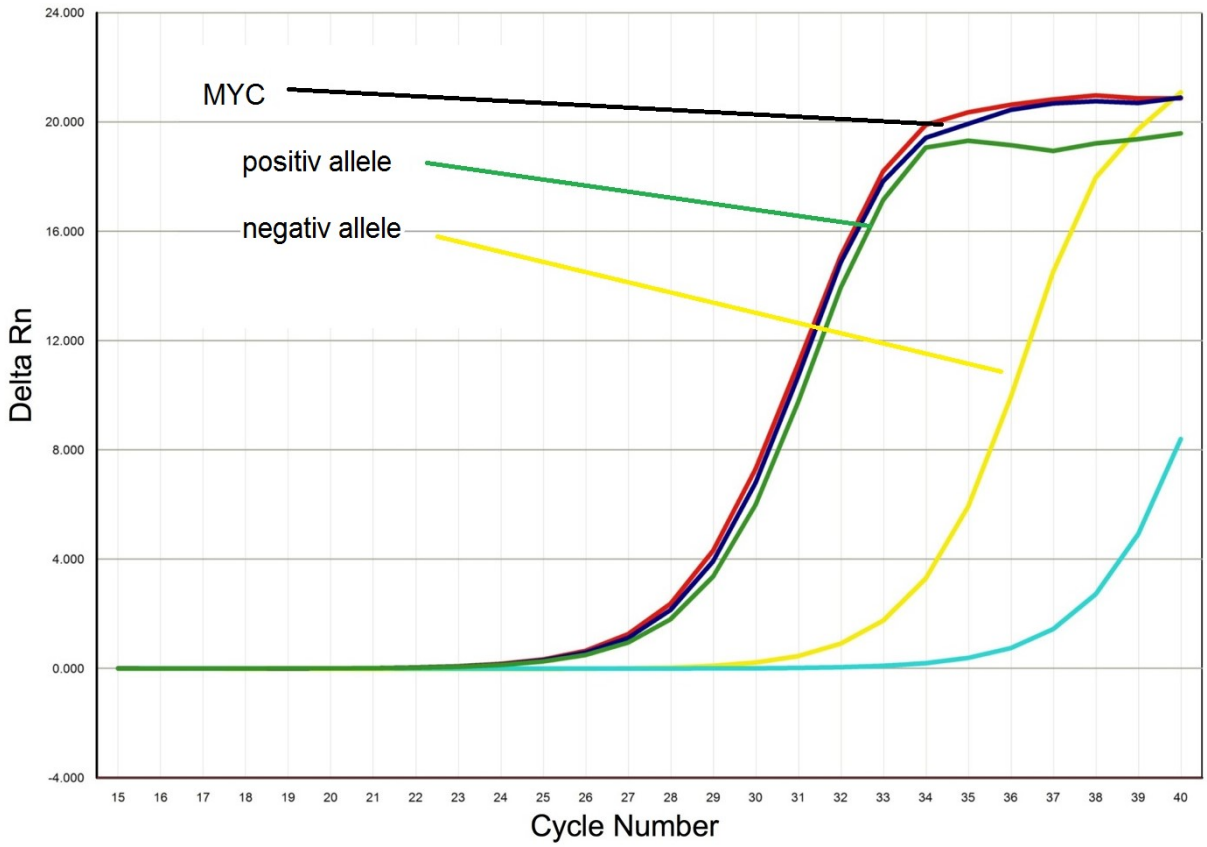

Figure 10. The two Homo c-MYC curves (one allele red and one allele blue) serve as a reference level. The green curve represents a SNP on one allele with a positive match; the yellow one represents a SNP on the other allele with no match. The ARMS primer will bind much faster on a positive match, therefore the qPCR curve of the negative match will be delayed.

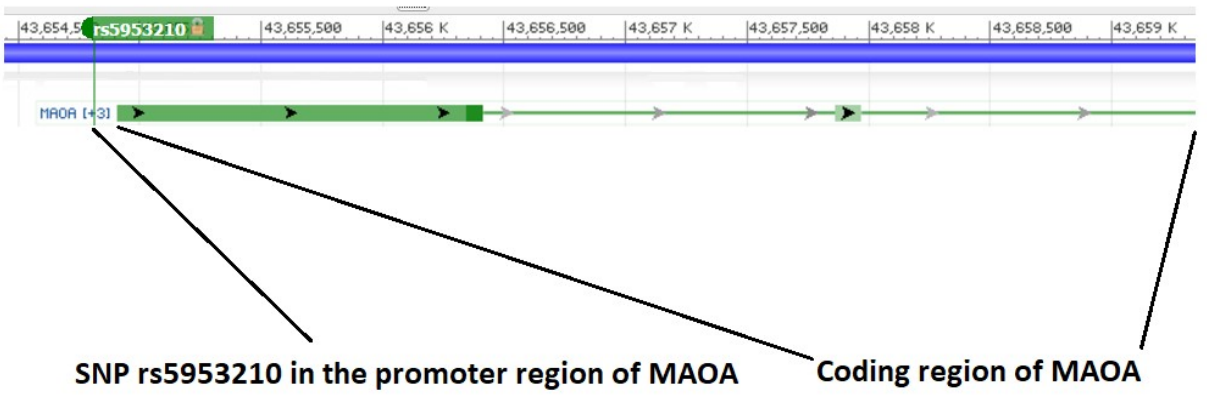

Figure 11. An observed SNP (rs5953210) in the promoter region of *MAOA* and the start of the coding region of *MAOA*.

5.3.1.1.1 Primer and probe design

SNP1 (rs57411356) is located in the promoter region of *MAOA* on position X: 43654560. One allele contains an adenine, the other allele a guanine. The primers were designed using Primer Express 2.0. First, File > New was selected. In the Type list, TaqMan Quantification was selected. The .doc files with the saved DNA sequence for SNP1 (see 5.3.1 SNP typing) was opened and the sequence was copied in the Sequence tab. Following parameters were changed in the program:
Amplicon Length: 50 to 200 bases
Primer Length: 13 to 25 bases
Tm: 55°C to 66°C
%GC: 20% to 80%
After selecting Tools > Find Primers all possible forward and reverse primers were listed in the Primers/Probes tab. After choosing a forward primer, we tried to find a reverse primer manual to match the melting temperature. The used forward primer had following sequence: TGAAAAACCCCACAGAGAACCAACA. The calculated melting temperature of the primer, which was determined by using Primer Express 2.0 was 64.5°C. The forward primer was the unspecific one and was used to amplify both alleles. The G-allele specific reverse primer had following sequence: ataCGGGGCGGGGAaGGG. The uppercase “G” is the SNP specific base and the lower case written “a” is the build in mismatch. An “ata” sequence on the 3-end of the primer was added to reach a melting temperature similar to the forward primer. The final primers melting temperature, calculated with Primer Express 2.0, was 64.8°C. The A-allele specific reverse primer had the same nucleotide sequence as the G-allele specific, but the guanine on the 17th position was exchanged with an adenine and the “ata” on the 3`end was changed to “aca”. This was done to reach an acceptable melting temperature of 64.1°C (calculated with Primer Express 2.0). The sequence of the reverse primer is as follow: acaCGGGGCGGGGAaGAG. The uppercase “A” is the SNP specific base and the lower case written “a” is the build in mismatch.
Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html) was used to design a probe to be used in the qPCR reaction to bind the amplicon. Another criterion was the length of the probe, which was chosen to be between 15 and 28

nucleotides. The melting temperature had to be at least 10°C above the elongation temperature. The ordered probe was marked with a FAM dye on the 5`end and the melting temperature was 76.5°C. The probe had following sequence:
[6FAM]TGCCTGGTCTCCCCCAAGTGACGG
The resulting amplicon had following nucleotide sequence (the nucleotide within the squared brackets presents the corresponding SNP):
TGAAAAACCCCACAGAGAACCAACAATTGCCTGGTCTCCCCCAAGTGACGGTTC
TCGCCCCGCCCCGTCCCGTCTCGCCTCGCCCCGCCCCTCCCCGCTCCTCCCC
GCCCCTCCCCGCTCCTCCCCGCCCCTCCCCGCTCCTCCCCGCCCCTCCCCGC
CCCTCCCCGCCCCTCCCCGC[C/T]CCTCCCCGCCCCG[ATA/ACA]
The last three nucleotides depend on the allele we were trying to amplify.
Mfold was used to calculate the expected secondary structure (Figure 12, a). The ionic concentration was changed to 50mM Na^+ and 2mM Mg^{++}. The T value was changed to 60°C. The calculated ΔG value was 0.19kcal/mol.

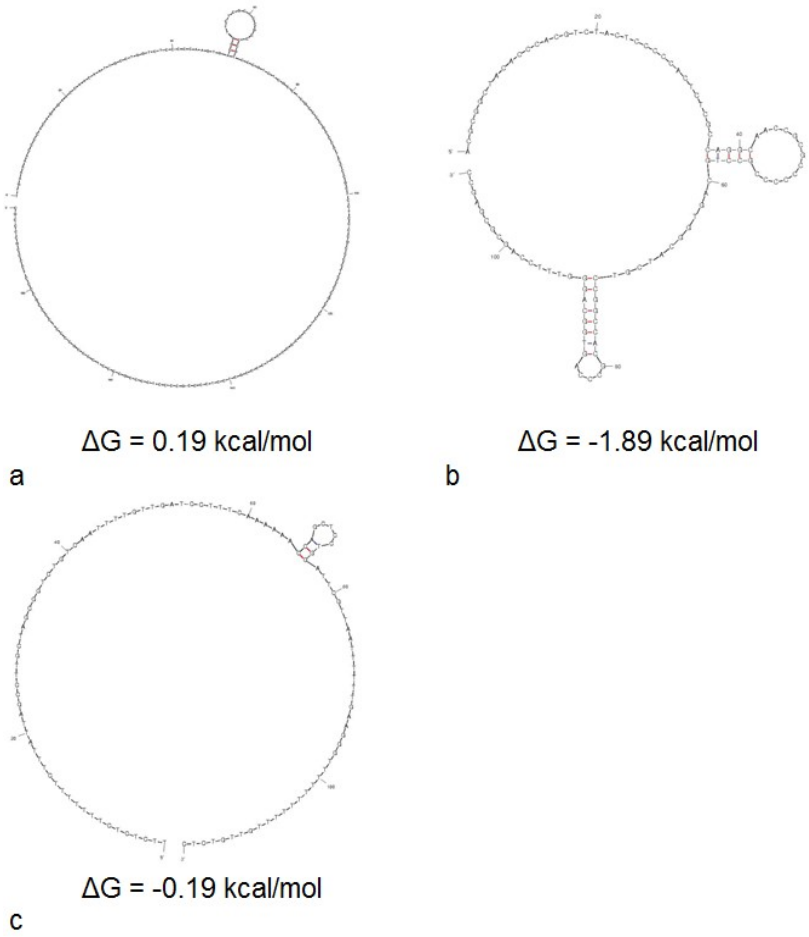

Figure 12. The secondary structure of the expected amplicon in the qPCR used to identify the genotypes of the SNPs. Picture a shows the secondary structure of the SNP 1 (rs57411356) amplicon, picture b of the SNP 2 (5953210) amplicon and picture c of the SNP 3 (rs79487021) amplicon. The ΔG value is the free energy in the system. A low value means a stronger secondary structure. Mfold was used to draw the structures and to calculate ΔG.

The second SNP rs5953210 is located in the promoter region of *MAOA* on position 43654798 on the chromosome X. One allele contains an adenine, the other allele a guanine. The primers were designed using Primer Express 2.0. First, File > New was selected. In the Type list, TaqMan Quantification was selected. The .doc files with the saved DNA sequence for SNP2 (see 5.3.1 SNP typing) was opened and the sequence was copied in the Sequence tab. Following parameters were changed in the program: Amplicon Length: 50 to 200 bases
Primer Length: 13 to 25 bases

Tm: 55°C to 66°C
%GC: 20% to 80%
After selecting Tools > Find Primers all possible forward and reverse primers were listed in the Primers/Probes tab. After choosing a reverse primer, we tried to find a forward primer manual to match the melting temperature. The unspecific reverse primer we used to amplify both alleles had following sequence: GGCTCGCGCTGGAAACC. Primer Express 2.0 was used to calculate the melting temperature of 61.4° C. The G-allele specific forward primer had following sequence: CGCGGCTACACCCtCGT. The uppercase “G” is the SNP specific base and the lower case written “t” is the build in mismatch. This primer sequence was chosen because of the melting temperature, calculated with Primer Express 2.0, was nearly the same as the melting temperature of the unspecific reverse primer, 59.4° C. The A-allele specific forward primer was the same as the G-allele specific, but the guanine was exchanged with an adenine. To reach a melting temperature similar to the other primers, an adenine was added on the 3´-end. The melting temperature with the added adenine, calculated with Primer Express 2.0, was 57.7° C. The primer had following sequence: ACGCGGCTACACCCtCAT. The uppercase “A” is the SNP specific base and the lower case written “t” is the build in mismatch.
Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html) was used to design a probe (see SNP1, Primer and probe design). The ordered probe was marked with a FAM dye on the 5`end and the melting temperature was 72.5°C. The probe had following sequence: [6FAM]CCCACTCTCGCCAGGCAACCGC
The resulting amplicon had following sequence, the marked nucleotide is the SNP:
CGCGGCTACACCCAC[G/A]TCTACTCCCCCACTCTCGCCAGGCAACCGCGCCCC
CCGCCTGCAGTGGCATCGTCCGGCCACGCCCAGTGGCAGGGTTTCCAGCGCG
AGCC
Mfold was used again (see SNP1, 5.3.1.1.1 Primer and probe design) to calculate the secondary structure of the amplicon (Figure 12, b).

The third SNP rs79487021 is located in an intron (intron 1) region of *MAOA* on position chrX: 43673345. One allele contains a cytosine, the other allele a thymine. The primers were designed using Primer Express 2.0. First, File > New was selected. In the Type list, TaqMan Quantification was selected. The .doc files with the saved

DNA sequence for SNP3 (see 5.3.1 SNP typing) was opened and the sequence was copied in the Sequence tab. Following parameters were changed in the program:
Amplicon Length: 50 to 200 bases
Primer Length: 13 to 25 bases
Tm: 55°C to 66°C
%GC: 20% to 80%
After selecting Tools > Find Primers all possible forward and reverse primers were listed in the Primers/Probes tab. After choosing a reverse primer, we tried to find a forward primer manual to match the melting temperature. An unspecific reverse primer with following sequence was used to amplify both alleles: ACAACAAAAAAAAAAACCCTTCA. The melting temperature, calculated with Primer Express 2.0, was 55.8° Celsius. As C-allele specific forward primer we had to use one with a similar melting temperature. The best matching primer sequence we found had following sequence: CTCTCTTTTTTTCTTTATTcGCC. The melting temperature, calculated with Primer Express 2.0 was 53.8° C. The uppercase “C” is the SNP specific base and the lower case written “c” is the build in mismatch. The T-allele specific forward primer was the same as the C-allele specific, but the cytosine was exchanged with a thymine. Two thymines were added on the 3´-end to reach a melting temperature compatible with the reverse primer. The melting temperature, calculated with Primer Express 2.0 was 55.8° C. The primer looks like follows: TTCTCTCTTTTTTTCTTTATTcGTC. The uppercase “T” is the SNP specific base and the lower case written “c” is the build in mismatch.
Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html) was used to design a probe (see SNP rs57411356, 5.3.1.1.1 Primer and probe design). The ordered probe was marked with a FAM dye on the 5`end and the melting temperature was 69.7°C. The probe had following sequence:
[6FAM]TGTTGATCCTTTCAAAAAACCAGCTCC
The resulting amplicon had following sequence, the marked nucleotide is the SNP:
CTCTCTTTTTTTCTTTATTAG[C/T]CTTGCTAGCGGTCTGTCAATTTTGTTGATCCT
TTCAAAAAACCAGCTCCTGGATTCGTTAATTTTTTGAAGGGTTTTTTTTTTTGTTG
T
Mfold was used again (see SNP rs57411356, Primer and probe design) to calculate the secondary structure of the amplicon (Figure 12, c).

To amplify the Homo c-MYC gene, a primer pair was designed using the program Primer Express 2.0. The primers were designed using Primer Express 2.0. First, File > New was selected. In the Type list, TaqMan Quantification was selected. The .doc files with the saved DNA sequence for the human c-MYC gene (see 5.3.1 SNP typing) were opened and the sequence was copied in the Sequence tab. Following parameters were changed in the program:
Amplicon Length: 50 to 150 bases
Primer Length: 13 to 25 bases
Tm: 58°C to 60°C
%GC: 30% to 80%
The following primer pair was used:
Forward primer: GGAACGAGCTAAAACGGAGCTT
Reverse primer: GGCCTTTTCATTGTTTTCCAACT
The melting temperature of the forward primer, was 59.9°C, the melting temperature of the reverse primer was 59.4°C.
Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html) was used to design a probe (see SNP rs57411356, 5.3.1.1.1 Primer and probe design). The ordered probe was marked with a HEX dye on the 5`end to distinct the c-MYC amplicons from the SNP amplicons, in which case the probes were labeled with a FAM dye. The melting temperature was 72.7°C. The probe had following sequence:
[HEX]TTGCCCTGCGTGACCAGATCCCG
The resulting amplicon had following sequence:
GGAACGAGCTAAAACGGAGCTTTTTTGCCCTGCGTGACCAGATCCCGGAGTTG GAAAACAATGAAAAGGCC

After the ordered primers and probes arrived, ddH_2O was added as described to get a concentration of 100 µM. The primers and probes were stored at -20°C.

5.3.1.1.2 qPCR

A qPCR, also called Quantitative Real-Time PCR, monitors the amplification of the target DNA during a PCR in real-time. To amplify DNA, a qPCR works in the same way as a standard PCR does. A DNA template, deoxyribonucleotides, at least one

pair of specific primers, a buffer solution and a thermo-stable DNA polymerase are used. A DNA-binding dye marked with a fluorophore is added to the PCR mixture. This dye binds to all double-stranded DNA (dsDNA) in the PCR, causing the dye to fluorescence. After each cycle, this effect is used to measure the amount of DNA product in the sample by measuring the increase of fluorescence intensity. However, the dye will also bind to nonspecific PCR products and primer dimer and affect the results of the measurement. Advantages of this method are the low costs because only one primer pair and no specific probe are needed. However, only one target can be observed per tube.

After designing all needed primers and a probe for each of the three SNPs, qPCR runs with all sample DNAs were done. We used this technique to determine if the used ARMS primer binds specific to the observed SNP. This will result in a much earlier rise of the amount of ds DNA in the PCR tube compared to a PCR reaction with the unspecific ARMS primer.

SNP rs57411356 qPCR

A great variety of different PCR protocols and qPCR mixes were tested to get usable results. Annealing temperature and elongation temperature were tested between 60° Celsius and 65° Celsius. We also tried different concentrations of DNA and adding so called S-solution. This S-solution should help to get better PCR results with GC rich amplicons. Each new qPCR protocol and qPCR mix was tested on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) operated by version 1.1 software. Six tubes (three tubes with “G” specific primers, three tubes with “A” specific primers, one of each tube was a negative controls without DNA but 2 µL TE buffer instead) were used for each run. To run the qPCR, 0.2mL PCR tubes were used. In each tube 2 µL 5xHOT FIREPol mix (EvaGreen® qPCR Mix Plus, Solis Biodyne), 0.25µL of each primer (10µM), one primer pair to amplify the SNP, one primer pair to amplify the c-MYC gene, 0.1µL SNP1 probe (10µM), 0.1µL c-MYC probe (10µM), 2 µL sample DNA (5ng/µL) and 6.8µL H_2O were pipetted. The tubes were mixed by inverting them several times. None of the tested protocols provided useable results. Consequently we decided only to test for SNP rs5953210 and SNP rs79487021.

SNP rs5953210 qPCR

As with SNP rs57411356 different protocols and qPCR mixes were tested. Annealing temperature and elongation temperature were tested between 58° Celsius and 65° Celsius. Because of the GC rich amplicon, adding S-solution was tried, which indeed provided better results than qPCR mixes without. Each new qPCR protocol and qPCR mix was tested on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) operated by version 1.1 software. Six tubes (three tubes with “G” specific primers, three tubes with “A” specific primers, one of each tube was a negative controls without DNA but 2 µL TE buffer instead) were used for each run. To run the qPCR, 0.2mL PCR tubes were used. In each tube 2 µL 5xHOT FIREPol mix (EvaGreen® qPCR Mix Plus, Solis Biodyne), 0.25µL of each primer (10µM), one primer pair to amplify the SNP, one primer pair to amplify the c-MYC gene, 0.1µL SNP2 probe (10µM), 0.1µL c-MYC probe (10µM), 2 µL sample DNA (5ng/µL), 1µL 10x S-solution and 5.8µL H_2O were pipetted. The tubes were mixed by inverting them several times. Following protocol provided the best results:

Table 2. The qPCR protocol to amplify SNP rs5953210.

qPCR protocol		
	95°C	t°C/time
1×	15 min	
65×	15 s	60/50 s

Table 3. Master mix for the PCR of SNP rs5953210.

qPCR master mix	volume [µL]	concentration [nM]
5x Hotfire	2	
forw rs (10µM)	0,25	250
rev rs (10µM)	0,25	250
Sonde rs (10µM)	0,1	100
forw Myc (10µM)	0,25	250
rev Myc (10µM)	0,25	250
Sonde Myc (10µM)	0,1	100
10x S-Solution	1	
H_2O	5,8	
overall	10	

Two 140x master mixes (one with a “G” specific primer, one with an “A” specific primer) were prepared by hand, to test the first 60 DNA samples, stored in the HCE210808-1 plate. 280µL 5xHOT FIREPol mix (EvaGreen® qPCR Mix Plus, Solis Biodyne), 35µL of each primer (10µM), one primer pair to amplify the SNP2, one primer pair to amplify the c-MYC gene, 14µL SNP2 probe (10µM), 14µL c-MYC probe (10µM), 140µL 10x S-solution and 532µL H_2O were pipetted into a 1.5mL Eppendorfer tube. To test each of our sample DNA, the liquid handling system epMotion® 5075 TMX (Eppendorf, Hamburg, Germany) was used for pipet the master mix and sample DNA together into a 96x96 well for the qPCR. Four times 2µL of the first 60 sample DNAs were pipetted from the HCE210808-1 plate into reaction tubes resulting in 240 tubes filled with sample DNA. In one half of the tubes (120) 8µL of the “G” specific master mix and in the other half of the tubes 8µL of the “A” specific master mix were pipetted resulting in two of each sample DNA mixed with “G” specific master mix and two of each sample DNA mixed with “A” specific master mix, respectively. The qPCR run was done on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) operated by version 1.1 software, using the PCR protocol seen in Table 2. Next, the same was done with the next 70 DNA samples (remaining 35 samples from plate HCE210808-1 and all 35 samples from plate HCE150908) using two new 180x master mixes and a new 96x96 well and also with the last 64 DNA samples (plate HCE061008) using two new 160x master mixes and a new 96x96 well.

SNP rs79487021 qPCR

In case of SNP rs79487021 none of the different amplification attempts (different PCR protocols, different annealing temperatures, PCR cycles and concentrations) provided useful results. To avoid unspecific primer binding a touchdown PCR was tested. A touchdown PCR starts with high annealing temperatures. This temperature is lowered with every few cycles. The primer will anneal at the highest tolerable temperature. At this high temperature, the primer will bind to the most specific region. This region will most likely be the region of interest. The resulting amplicons will be further amplified during the following cycles with lower temperature. Duo to the exponential nature of the PCR, the first amplified fragments will out compete the nonspecific sequences to which the primer may possible bind at the lower temperature cycles. Each new qPCR protocol and qPCR mix was tested on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) operated by version 1.1 software. Six tubes (three tubes with “C” specific primers, three tubes with “T” specific primers, one of each tube was a negative controls without DNA but 2 µL TE buffer instead) were used for each run. To run the qPCR, 0.2mL PCR tubes were used. In each tube 2 µL 5xHOT FIREPol mix (EvaGreen® qPCR Mix Plus, Solis Biodyne), 0.25µL of each primer (10µM), one primer pair to amplify the SNP, one primer pair to amplify the c-MYC gene, 0.1µL SNP2 probe (10µM), 0.1µL c-MYC probe (10µM), 2 µL sample DNA (5ng/µL), 1µL 10x S-solution and 5.8µL H2O were pipetted. The tubes were mixed by inverting them several times. Following touchdown protocol provided the best results:

Table 4. qPCR protocol to amplify SNP rs79487021.

qPCR protocol		
	95°C	t°C/time
1×	15 min	
2×	15 s	68/20 s
2×	15 s	67/20 s
2×	15 s	66/20 s
2×	15 s	65/20 s
2×	15 s	64/20 s
2×	15 s	63/20 s
2×	15 s	62/20 s
2×	15 s	61/20 s
55×	15 s	60/50 s

Table 5. Master mix for the PCR of SNP rs79487021.

qPCR Mix	volume [µL]	concentration [nM]
5x Hotfire	2	
forw rs (10µM)	0,25	250
rev rs (10µM)	0,25	250
Sonde rs (10µM)	0,1	100
forw Myc (10µM)	0,25	250
rev Myc (10µM)	0,25	250
Sonde Myc (10µM)	0,1	100
10x S-Solution	1	
H_2O	5,8	
overall	10	

Two 140x master mixes (one with a “C” specific primer, one with a “T” specific primer) were prepared by hand, to test the first 60 DNA samples, stored in the HCE210808-1 plate. 280µL 5xHotfire mix (EvaGreen, Solis Biodyne), 35µL of each primer (10µM), one primer pair to amplify the SNP3, one primer pair to amplify the c-MYC gene, 14µL SNP3 probe (10µM), 14µL c-MYC probe (10µM), 140µL 10x S-solution and 532µL H_2O were pipetted into a 1.5mL Eppendorfer tube. To test each of our sample DNA the liquid handling system epMotion® 5075 TMX (Eppendorf, Hamburg, Germany), was used for pipet the master mix and sample DNA together into a 96x96 well for the qPCR. Four times 2µL of the first 60 sample DNAs were pipetted from the HCE210808-1 plate into reaction tubes resulting in 240 tubes filled with sample DNA. In one half of the tubes (120) 8µL of the “C” specific master mix and in the other half of the tubes 8µL of the “T” specific master mix were pipetted resulting in respectively two of each sample DNA mixed with “C” specific master mix and two of each sample DNA mixed with “T” specific master mix. The qPCR run was done on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) operated by version 1.1 software, using the PCR protocol seen in Table 4. As next, same was done with the next 70 DNA samples (remaining 35 samples from plate HCE210808-1 and all 35 samples from plate HCE150908) using two new 180x master mixes and a new 96x96

well and also with the last 64 DNA samples (plate HCE061008), using two new 160x master mixes and a new 96x96 well.

5.3.2 VNTR typing

To calculate the numbers of repeats in the MAOA-uVNTR, the region was first amplified and sequenced at LGC Genomics, Ostendstrasse 25, Berlin, Germany. This was done with 48 male samples (only 45 worked) whereas only one allele (there is only one MAOA-uVNTR in the male genome, because there is only one X chromosome) would be amplified for each sample. The data were used as a reference point and to assign an amplicon with a given length to the right number of repeats. As next, all samples were amplified and the amplicons marked with a dye using a method called M13 tailing. The used primers were ordered at Sigma-Aldrich Handles GmbH, Marchettigasse 7/2, 1060 Wien, Austria. The amplicons lengths were determined using an Agilent 2100 Bio analyzer for Restriction Fragment Length Polymorphism (RFLP) Analysis at LGC Forensics, Stolberger Straße 370, Köln, Germany.

5.3.2.1 M13 Tailing

A nested PCR method was used to amplify the promoter region of *MAOA* encompassing the VNTR locus. The labeling of the PCR product was necessary to determine the length of the PCR fragment. With the given length, we were able to calculate the exact number of repeats in the *MAOA* promoter region.
Three different primers were used to amplify and label the promoter region. One of the primers was a M13 tail labeled with a fluorescence dye. In this experiment 6-carboxy-fluorescine (FAM) was used. The forward primer was a sequence specific primer with an M13 tail on its 5´ end. The reverse primer was a sequence specific primer without any modifications. Different concentrations of primers and different annealing temperatures during the PCR were used to get amplicons marked with FAM on its 5´end. The concentration of the forward primer was 60nM, the concentration of the reverse primer was 260nM and the concentration of the M13 tail primer was 200nM. In addition, the conditions of the first cycles of the PCR were chosen to favor the forward primer with the M13 tail over the labeled M13 tail primer.

This was accomplished by using a higher annealing temperature of 63°C. The forward primer with the M13 tail is incorporated into the PCR products (Figure 13, a). After 35 cycles all of the forward primer was depleted and the annealing temperature was lowered for the last 8 cycles to favor the M13 tail primer (Figure 13, b). The FAM labeled primer acts as the new forward primer and incorporates the FAM label into the PCR product (Figure 13, c).

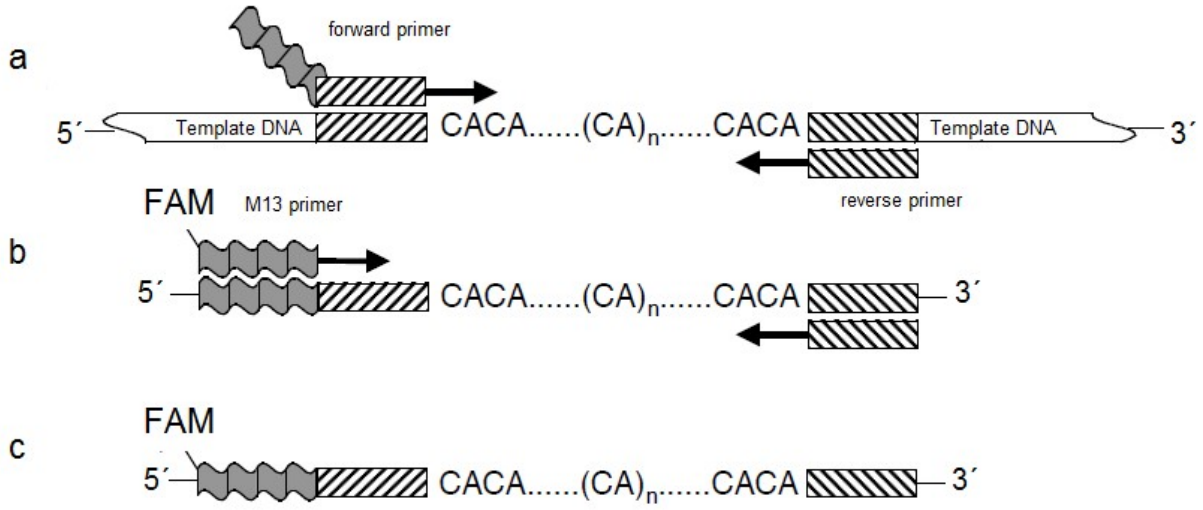

Figure 13. M13-tailing PCR method. (a.)The M13 tail becomes incorporated in the first 35 PCR cycles. (b.) After 35 cycles the forward primer is depleted and the annealing temperature is lowered to favor the FAM marked M13 primer. (c.) The FAM label finally is incorporated into the PCR product. Adapted from Schuelke et al. 2000.

5.3.2.1.1 Primer design

To amplify the pVNTR region of the *MAOA* gen, nearly the same primers were used as described in Zhu et al. (1992). The forward primer used in Zhu et al. (1992) has following sequence: 5'-ACAGCCTGACCGTGGAGAAG-3'. To obtain an optimal melting temperature which is approximately 10°C higher as the M13 primer, "AGA" was added to the 5´-end of the primer. In addition, also the M13 sequence was added on the 5´-end. The melting temperature of the primer, calculated with Primer Express 2.0, was 63°C. The final forward primer (including M13 tail marked as underlined sequence) had following sequence:
TGT AAA ACG ACG GCCAGTAGAACAGCCTGACCGTGGAGAAG.

The reverse primer used in Zhu et al. (1992) had following sequence: 5'-GAACGGACGCTCCATTCGGA-3´. The first "G" on the 5´-end was deleted to get a melting temperature of 62.4°C which is similar to the melting temperature of the forward primer. Primer Express 2.0 was used again to calculate the melting temperatures. Furthermore, "GTTT" was added on the 5´end to prevent the Taq polymerase from catalyzing the addition of non-template nucleotides. This can happen sometimes on primers with A, C or G on their 5´end (Brownstein et al 1996). The final primer had following sequence:
GTTTAACGGACGCTCCATTCGGA.
The M13 primer had following sequence:
FAM-TGTAAAACGACGGCCAGT.
The primer was FAM labeled on the 5´-end in order to use ABI machines to calculate the length of the amplicons and furthermore to calculate the number of repeats in the MAOA-uVNTR.

5.3.2.1.2 PCR protocols for sequencing and RFLP

All male samples from the plate HCE150908 (20 male samples) and the plate HCE061008 (28 male samples) were sequenced at LGC Genomics, Ostendstrasse 25, Berlin, Germany. The PCRs were carried out in a final volume of 20µl in 0.2mL PCR tubes. Each PCR tube contained 2µL 5xHotfire mix (EvaGreen Super Plus, Solis Biodyne), 0.52µL of the forward primer with the M13 tail (10µM), 0.52µL of the reverse primer (10µM), 2µL genomic DNA (5ng/µL) and 12.96µL distillated H_2O. The pipetting was done by hand. To mix it, the tubes were inverted several times. In addition to the 48 samples one negative control containing 2µL TE instead of DNA was made. The PTC-200 Peltier Thermal Cycler (MJ Research) was used to run the PCR. Following PCR protocol was used:

Table 6. PCR protocol to amplify the *MAOA gene*.

	t°C/time	t°C/time	t°C/time
1x	95/15 min		
40x	95/30 s	63/40 s	72/40 s
1x	72/15 min		

Subsequently the PCR products were purified as follows: all 48 samples were stained with EvaGreen and subsequently loaded on a 1% agarose gel with a molecular marker; the electrophoresis condition were 180V for 25 minutes; the DNA bands were cut out and transferred into 1.5mL tubes. Hi Yield® Gel/PCR DNA Fragment Extraction Kits (Süd-Laborbedarf GmbH) was used for purification. After the purification step, 12µL of each sample and 2µL of the reverse primer (10µM) were pipetted into a new 1.5mL tube and sent to LGC Genomics, Ostendstrasse 25, Berlin, Germany for sequencing.

For the restriction fragment length polymorphism (RFLP) experiment, different restriction enzymes were used to cleave the sample DNA at specific nucleotide sequences. After the digesting, an agarose gel is used to separate the DNA fragments according to their size.
First a 78x master mixes was prepared by hand, to amplify the first 33 DNA samples, stored in the HCE061008 plate. 312µL 5xHOT FIREPol mix (EvaGreen® qPCR Mix Super Plus, Solis Biodyne), 9.36µL of the forward primer (10µM), 4.56µL of the reverse primer (10µM) , 31.2µL of the M13 primer (10µM) and 1010.88µL H_2O were pipetted into a 1.5mL Eppendorfer tube an mixed by inverting the tube several times. The liquid handling system epMotion® 5075 TMX (Eppendorf, Hamburg, Germany), was used to pipet the master mix and sample DNA together into 0.2mL reaction tubes for the PCR. Two times 2µL of the first 33 sample DNAs and 2 negative controls (containing 2µL TE buffer instead of sample DNA) were pipetted from the HCE061008 plate into reaction tubes resulting in 68 tubes filled with samples. The PCR run was done on a PTC-200 Peltier Thermal Cycler (MJ Research), using the PCR protocol as shown in Table 7. Next, same was done with the next 21 DNA samples (from plate HCE150908) using a new 52x master mixes and also with the last 42 DNA samples (plate HCE210808-1) using a new 100x master mixes.

Table 7. PCR protocol for the RLFP amplification.

PCR Protokoll			
	95°C	t°C/time	t°C/time
1×	15 min		
35×	30 s	63/40 s	72/40 s
8x	30 s	53/40 s	72/40 s
1x		72/15 min	

After the PCR runs, 1% agarose gel was made and all 198 samples (2x96 samples + 6 negative controls) were loaded on the gel. The DNA bands were cut out and transferred into 1.5mL Eppendorfer tubes. Hi Yield® Gel/PCR DNA Fragment Extraction Kits (Süd-Laborbedarf GmbH) was used for purification. The samples were sent to LGC Forensics, Stolberger Straße 370, Köln, Germany for length determination.

5.4 Linkage disequilibrium between SNPs and MAOA-uVNTR

To simplify the identification of the MAOA-uVNTR genotype in further experiments, we tried to test for linkage between the MAOA-uVNTR which influences the expression level of *MAOA* and specific SNPs within the *MAOA* gene region. SNP typing is a more reliable molecular typing approach than assessing the VNTR at this locus. Three different SNPs which are most likely to be linked to the MAOA-uVNTR were tested: SNP rs57411356 in the promoter region of *MAOA* (chrX: 43513807), SNP rs5953210 also in the promoter region of *MAOA* (chrX: 43514045) and SNP rs79487021 which is located in the intron 1 of *MAOA* gene (chrX: 43673345). The genotypes of the alleles (SNP alleles and MAOA uVNTR alleles) were identified as described in the subsection “5.3.1 SNP typing” and “5.3.2 VNTR typing”. As a result, usable genotype data could only be obtained from one SNP, rs5953210. An X^2 test was carried out to evaluate the probability that the observed genotypes (combination of SNP rs5953210 genotype and the MAOA-uVNTR genotype) arouse by chance. To test for a LD, the deviation (D) in the observed frequency of haplotypes from the expected was calculated (Table 8). We also calculated the normalized D, D’, by dividing it by the theoretically maximum (D_{max}) for the observed allele frequency (Table 8). In our case (D>0) D_{max} is the smaller of p_1q_2 and p_2q_1.

Table 8. Formals to calculate the deviation (D) and the normalized deviation (D').

Allele frequency		Haplotype frequency				
					G	A	
				3,5	x11=p1*q1+D	x21=p2*q1-D	q1
G	p1=0,2152	G/VNTR 3,5	x11=0,1772	4,5	x12=p1*q2-D	x22=p2*q2+D	q2
A	p2=0,7848	G/VNTR 4,5	x12=0,0380		p1	p2	
VNTR 3,5	q1=0,3038	A/VNTR 3,5	x21=0,1266				
VNTR 4,5	q2=0,6962	A/VNTR 4,5	x22=0,6582				
p1*q2= 0,1498					D=x11*x22-x12*x21	**D=0,1118**	
p2*q1=0,2384					D´=D/Dmax	**D´=0,7465**	

As shown in Table 8 there exists a LD between the two loci (MAOA-uVNTR and SNP rs5953210).

5.5 1000 Genome data

The 1000 Genome Project data base (http://www.internationalgenome.org/) was used to download information about the distribution of the SNP rs5953210 (chromosome X: 43.654.798) which is located within the *MAOA* promoter. The gathered information of the SNP distribution in couples was added to our own samples to increase the sample size to work with.

5.5.1 SNP rs5953210

To obtain genotype data of the rs5953210 SNP from additional samples we used the 1000 Genome Project database. Only phase 3 data from the 1000 Genomes Project (*http://phase3browser.1000genomes.org*) were used. 107 samples from the Iberian population in Spain (IBS) were downloaded and saved in an excel table. In the first row, the samples IDs were written and in the second row the matching SNP allele. The new allele data were added to our own allele data we already got from our samples.

5.6 Data analysis and statistical analysis

The spreadsheet program Microsoft Excel was used to calculate the MHC distances, the SNP distribution of rs5953210, the linkage disequilibrium (LD) between the MAOA-uVNTR and the SNP rs5953210 and the link between mate choice and *MAOA* genotype.

5.6.1 Mate choice depending on HLA distances

Microsoft Excel was first used to calculate the average distances of each individual HLA allele and the average distance of the whole HLA genotype between each individual and between the two partners of every married couple. Therefore a table with each sample ID and the three HLA alleles, HLA A, HLA B and HLA DR1, was created. To each sample ID six HLA genotypes were assigned, 2 genotypes from each allele. As next, the HLA alleles (A, B and DR1) from every sample were compared to each other to calculate the average distances of each of the three HLA alleles. If two individuals shared none HLA allele, the distance is 0, if one HLA allele is shared, we assumed the distance as 1, and if both alleles are shared the distance is 2. The same was done to calculate the distance between the whole HLA genotype. In this case the distance is 6 if every HLA allele matched and 0 if none HLA allele matched. Next, the average distances of individuals and couples were compared to find a preference in dissimilarity or similarity regarding the MHC allele in the mate choice. The Standard deviations of the couples were calculated on each allele and over the whole HLA genotype. The statistic program R (https://www.r-project.org/) was used to calculate the p-values and check if there is any significance ($p<0.05$). For that reason, the distances of every individual and every couple from each HLA allele were imported and a two sided t-test was done.

5.6.2 rs5953210 and VNTR distribution

To examine the distribution of the SNP rs5953210 in the female population, our own samples and the downloaded data from the 1000 Genomes data base were used. The distribution of the MAOA-uVNTR in females was also calculated. First, Microsoft Excel was used to calculate the allele frequency of the SNP and the VNTR in the whole female population. The Hardy Weinberg equilibrium (HWE) was used to

calculate the expected allele frequency in the female samples. Next a Pearson´s chi-square statistic (X^2) test was done using R (https://www.r-project.org/). This test evaluates how likely it is, that an observed difference of two sets of data arose by chance. Each of the observed events has to be mutually exclusive with a total probability of 1. The X^2 value was calculated by comparing the calculated allele frequency with the observed allele frequency in the female samples. If the null hypothesis is true, the distributions of the observed events in the samples are consistent with the calculated distribution.

5.6.3 LD between Monoamine oxidase A-uVNTR and rs5953210

Microsoft Excel was used to calculate the linkage disequilibrium (LD) between the SNP rs5953210 and the MAOA-uVNTR in our samples. To test for a LD, the deviation (D) and D prime (D´) were calculated. The LD is a deviation in the observed frequency of a haplotype from the expected frequency. A deviation in the expected frequency ($D \neq 0$) means, that the two compared loci are in LD. The value of D is in a range between -0.25 and +0.25. To normalize D, it is divided by the theoretical maximum for the observed allele frequency. This normalized D is called D`. To do so, the allele frequencies of two genotypes of the VNTR (the VNTR with 3.5 repeats and the VNTR with 4.5 repeats) and the allele frequency of the SNP rs5953210 were calculated. Next the haplotype frequencies of the four possible combinations (x_{11}, x_{12}, x_{22}, x_{21}) were calculated by multiplying the allele frequencies. D and D´ were calculated using following formulas:

$D=(x_{11})*(x_{22})-(x_{12})*(x_{21})$, $D`=D/D_{max}$

5.6.4 Mate choice depending on MAOA genotype

First the genotype frequency of the MAOA-uVNTR with 3.5 and 4.5 repeats in our male samples was calculated using Microsoft Excel. The females were separated in 3 different groups depending on their VNTR genotype. The first group was homozygote with the VNTR with 3.5 repeats, the second group was heterozygote (one allele with the VNTR with 3.5 repeats the other with 4.5 repeats) and the third group was homozygote with the VNTR with 3.5 repeats. Next the distributions of the

VNTR genotype of the chosen partners for each of the three groups were calculated. Then, the distributions of the chosen partners were compared with the allele distribution in the male samples to check for a linkage. This was done with the statistic program R (https://www.r-project.org/). After importing the data a X^2 test was done (see ”5.6.2 rs5953210 and VNTR distribution”) to calculate the *p*-values and to check for any significance.

6. Results

6.1 HLA genetic diversity/typing

We used sequence-specific primer (SSP) technology and obtained the genotypes of the HLA class I genes HLA-A, HLA-B and HLA-C and the HLA class II gene HLA-DRB1 in 102 humans (51 married couples). The supplementary file Table 1 presents the allelic diversity obtained in our studied samples.

6.2 MAOA genetic diversity/typing

We analyzed three different SNPs (rs57411356, rs5953210 rs7948702) to determine the *MAOA* genotypes in 158 samples. However, only the SNP rs5953210 could be used in the subsequent analyses because the other two SNPs (rs57411356 and rs7948702) resulted in non-reproducible PCR products despite of using many different qPCR protocols.

6.2.1 MAOA SNP typing

We successfully obtained results of the SNP rs5953210 and were able to determine the SNP in 158 samples. The data were used to calculate the distribution of the SNP rs5953210 in females and to test for a LD between SNP rs5953210 and MAOA-uVNTR. A spreadsheet with the sample IDs, couple IDs (only if the sample is from a couple) and the corresponding genotype can be found in the supplementary files (supplementary file: Table 2). We could not obtain any results in the qPCR runs using ARMS primer to determine the genotype of the other analyzed SNP rs57411356. Despite using many different PCR protocols none of them accomplished to amplify the sample DNA containing the SNP. The SNP rs7948702 was successfully determined but double checking of the results showed, that some cases with two positive results (positive C and positive T) in male samples (which should be impossible because there is only one X chromosome in males) occured. In addition, nearly all tested samples were tested positive for the T allele. Thus, we checked whether the DNA sequence we amplified also occurs on other regions of the human genome. We found that the intron sequence containing the SNP rs7948702 is also

present on chromosome 9. Consequently, we decided not to use the results of this SNP.

6.2.2 MAOA VNTR typing

The variable number tandem repeat in the MAOA-uVNTR of 143 individuals (28 couples) could be determined. In the literature, four different alleles with different numbers of repeats are reported: 3, 3.5, 4 and 5 repeats. However, in our own experiment, we observed following number of repeats: 2.2, 2.5, 3.5, 4.2, 4.5 and 5.5. This differing results could be the consequence of a newer method we applied to analyze the DNA samples. The majority of the samples are either carrying the 3.5 repeat polymorphism (46.4%) or the 4.5 repeat polymorphism (60.1%). Alleles with 2.5 and 5.5 repeats were not included in the calculations because the allele frequency was too low (<1%). A spreadsheet with the sample IDs, couple IDs (only if the sample is from a couple) and the corresponding alleles can be found in the supplementary files (supplementary file: Table 3.)

6.3 Statistic analysis

6.3.1 SNP rs5953210 and Monoamine oxidase A-uVNTR distribution

Figure 14 and Figure 16 show the calculated allele frequency of the SNP rs5953210 and the MAOA-uVNTR in the female population in comparison to the whole population. We found no significant difference (X^2=1.001, *p*(df=2)=0.60) between the expected and observed frequency (Figure 15, Figure 17).

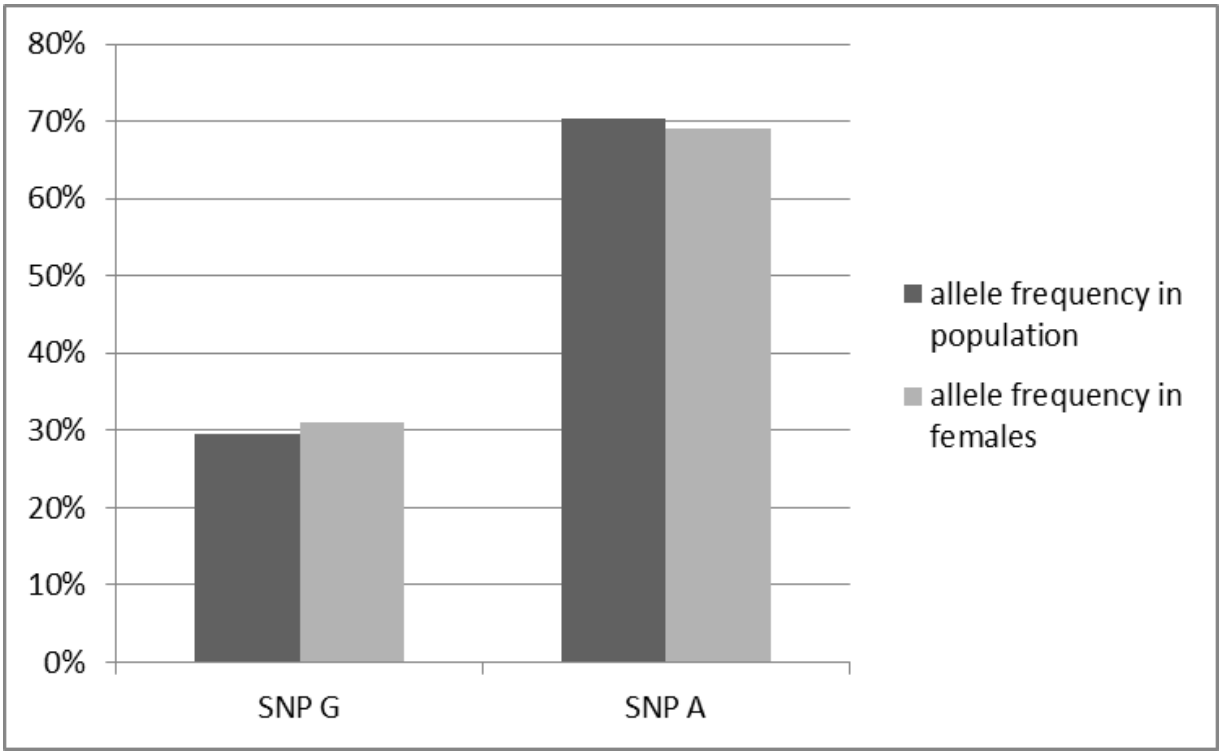

Figure 14. A comparison of the allele frequency between the whole population and the female population.

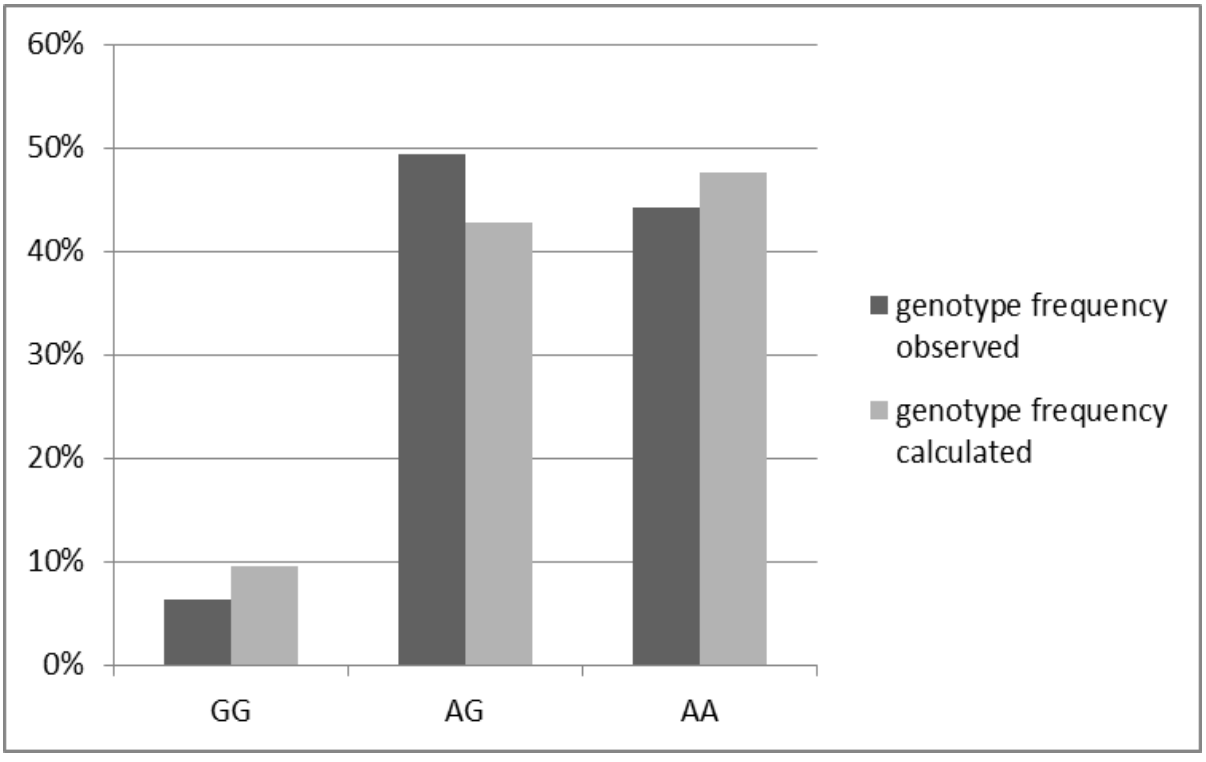

Figure 15. A comparison between the observed and the expected genotype frequency of SNP rs5953210 in the female population. X^2=1,001, *p*(df=2)=0,6062.

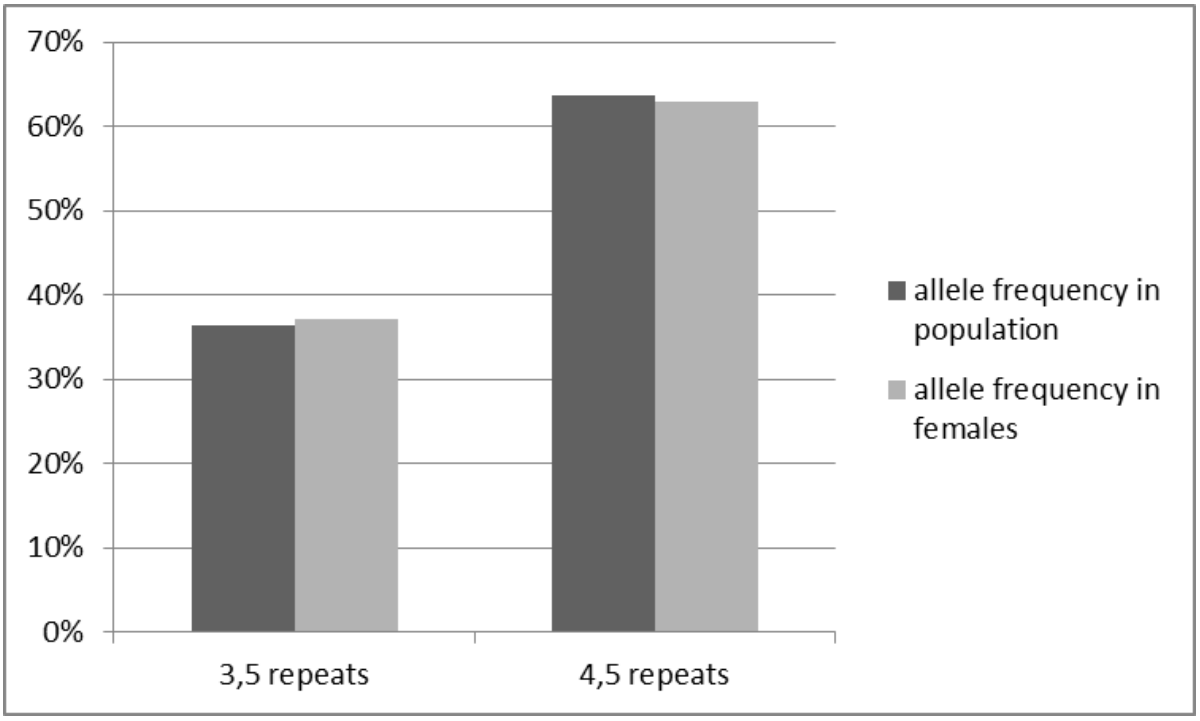

Figure 16. A comparison of the MAOA-uVNTR (3.5 repeats and 4.5 repeats) frequency between the whole population and the female population.

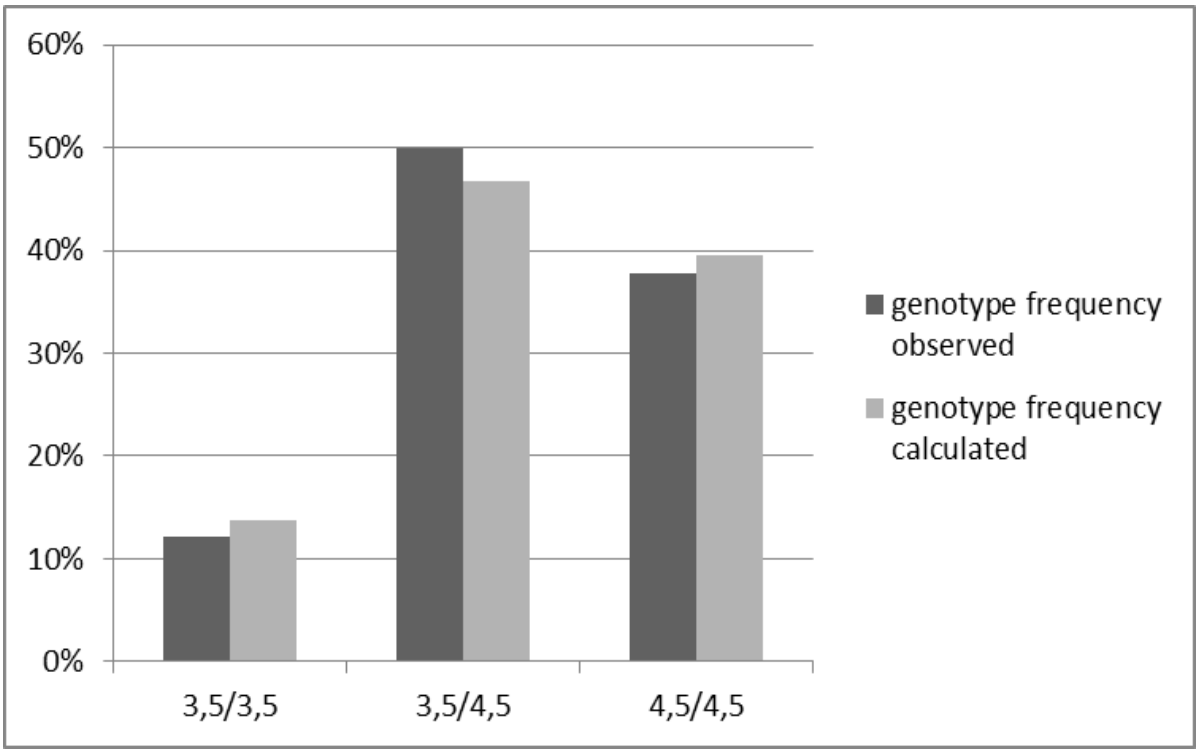

Figure 17. A comparison between the observed and the expected genotype frequency of the MAOA-uVNTR in the female population. X^2=0.1867, *p*(df=2)=0.9109.

We found no significant difference in the observed distribution of the SNP rs5953210 or the MAOA-uVNTR and the calculated (expected) distribution. The *p*-values of the SNP distribution is *p*(df=2)=0,6062 and the *p*-value of the VNTR distribution is *p*(df=2)=0,9109.

6.3.2 LD of Monoamine oxidase A-uVNTR and SNP rs5953210

A significant association between the SNP and the VNTR was found. The calculated X^2 was 31.959 and the *p*-value (df=6) was 0.01. The experiment showed if the SNP nucleotide is an A, the number of repeats in the VNTR is most likely 4.5. If the nucleotide is a G, the number of repeats is most likely 3.5 and therefor *MAOA-H*. We calculated D and D': D=0.1118, D´=0.7465.

6.3.3 Mate choice dependence on Monoamine oxidase A-uVNTR genotype

We tested whether mate choice in humans is associated with the MAOA-uVNTR allelic variation. Therefore we compared the allele frequency of the VNTR with 3.5 and 4.5 repeats in the human male population with the allele frequency in all couples (Figure 18). The married couples were sorted by the female genotype of the VNTR in three groups. Two groups were homozygote for the VNTR allele (females with 3.5 or 4.5 repeats) and one group was heterozygote for the VNTR allele (females with 3.5 repeats in one allele and 4.5 repeats in the other allele).

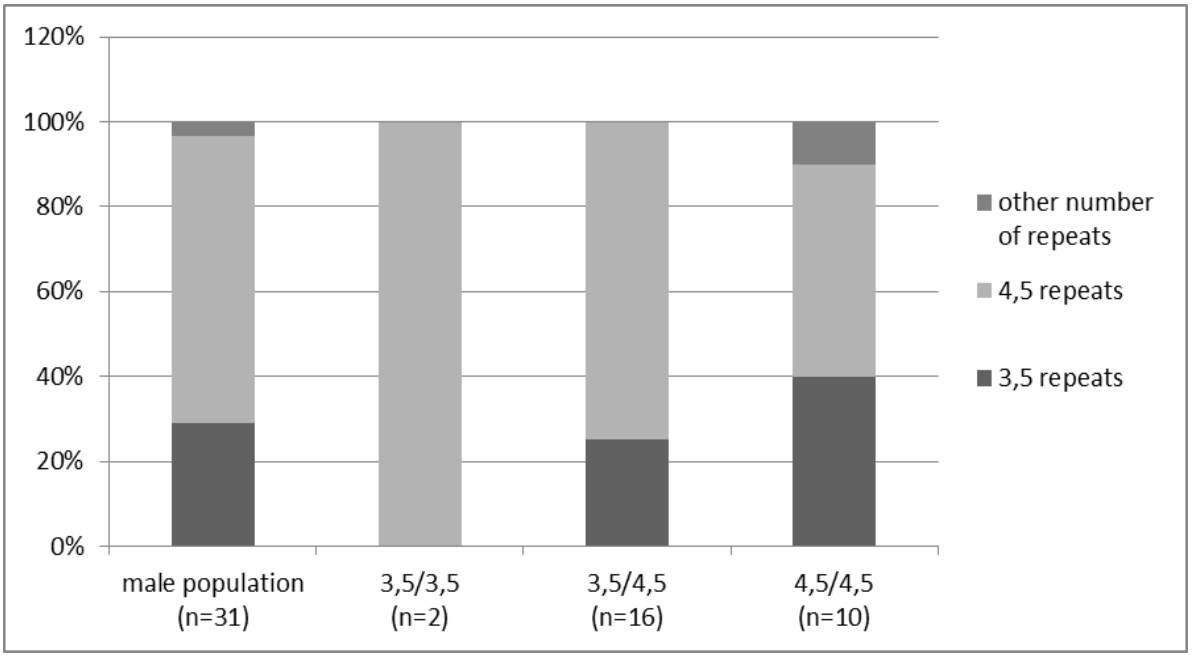

Figure 18. A comparison between the allele frequency of males in the whole population and the allele frequency of males in married couples. The first row displays the allele frequency of males in the population, the second row in couples with females with the VNTR genotype with 3.5/3.5 repeats, the third row in couples with females with the VNTR genotype with 3.5/4.5 repeats and the fourth row in couples with females with the VNTR genotype with 4.5/4.5 repeats.

We found no significant difference in the allele frequency of the MAOA-uVNTR of males within a relationship and single males. Furthermore, we found no significant difference in the allele frequency of males within the four groups. The X^2-value for all four groups is 3.889 and $p(df=6)=0.6917$. The calculated X^2-values in the first group (males in relationship with 3.5/3.5 women) were: $X^2=0.92567$, $p(df=2)=0.6295$. The values in the second group (males in relationship with 3.5/4.5 women) were: $X^2=0.65734$, $p(df=2)=0.7199$ and the values in the third group (males in relationship with 4.5/4.5 women) were: $X^2=1.3734$, $p(df=2)=0.5032$. However, the *p*-values are not absolute reliable because not all sample sizes in the calculation are five or higher.

6.3.4 Mate choice and MHC genetic distances

To test if the MHC genotype influences human mate choice, we calculated the average MHC genetic-distances within a population (102 individuals) and within couples (51 couples) of HLA-A, HLA-B and HLA-DRB1. The same was done with the

HLA genotype in total (Figure 19). The calculated distances for the HLA genotypes of each individual and each couple can be found in the supplementary files (supplementary file: Table 4).

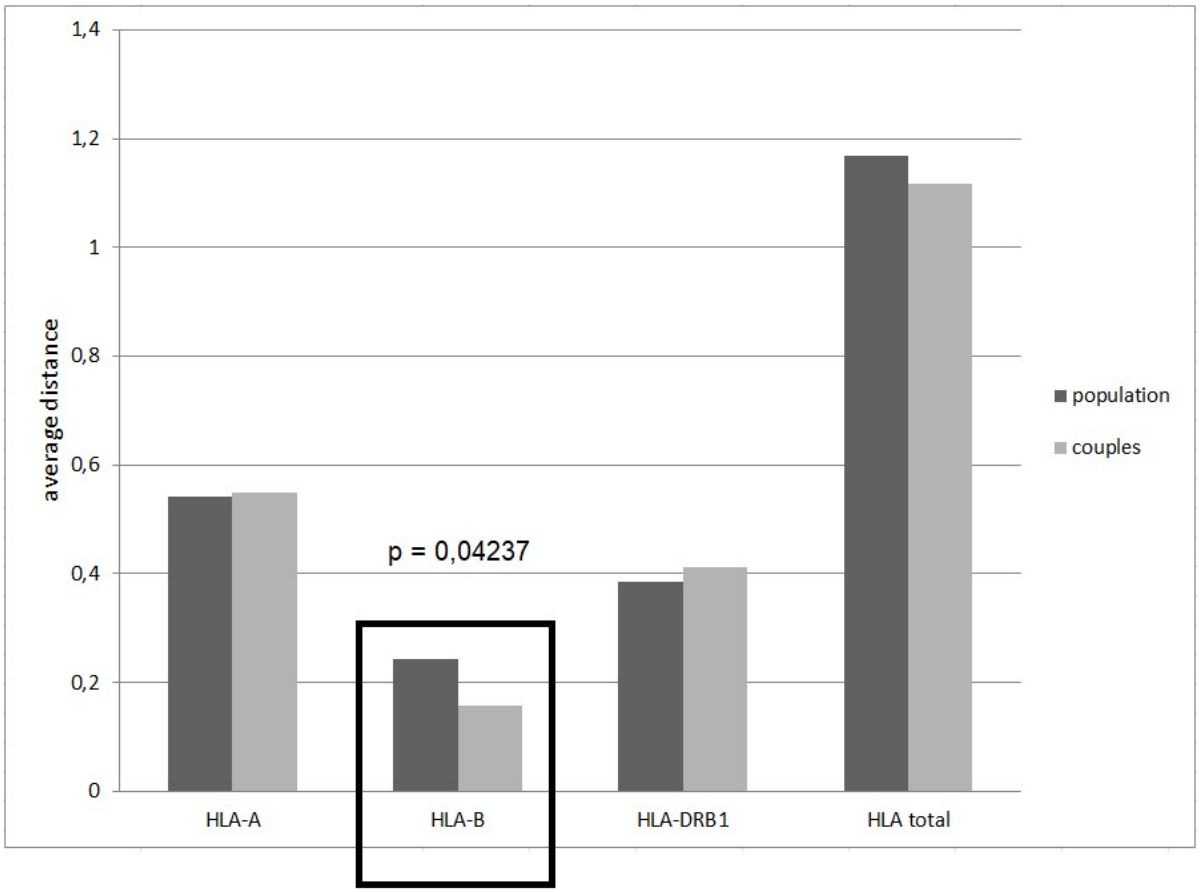

Figure 19. Average distances of the HLA genotypes within the total population and within couples. HLA-B with a significant *p*-value is highlighted.

The statistical analysis resulted in significant *p*-values (0.04237) for the HLA-B gene. The other tested HLA genes (HLA-B, HLA-DR1) separately and combined were non-significant ($p > 0.05$).

7. Discussion

In this study we analyzed the influence of genes on the human pair bonding behavior. We aimed to identify whether SNPs in the *MAOA* gene and the MAOA-uVNTR genotype affect the partner choice in humans. We also studied the HLA genotype diversity in 51 married couples and analyzed whether the genetic diversity at the HLA loci influenced pair bonding in humans. The obtained data were also used to identify possible linkage disequilibrium between the SNP rs5953210 and the number of repeats in the MAOA-uVNTR.

7.1 Number of repeats in the MAOA u-VNTR

Studies showed that the MAOA u-VNTR polymorphism with 3.5 and 4 repeats has an optimal length leading to an optimal expression of *MAOA* (*MAOA-H*). The 3R and 5R alleles are either too short or too long and both leading to a low expression of *MAOA* (*MAOA-L*) (Sabol et al., 1998). The polymorphism we detected in our samples had 2.2, 2.5 3.5, 4.2, 4.5 or 5.5 repeats. The 3.5R is known and mostly leads to an optimal expression of *MAOA*; however, the other alleles (2.2, 2.5, 4.2, 4.5 and 5.5) weren't described until now. The length of the 4.2R allele and the 4.5R allele lies between the 4R (*MAOA-H*) and the 5R (*MAOA-L*) allele. This leads to the conclusion that these two alleles could either be in the range of having an optimal length to act as an upstream activator for *MAOA* (MAOA-H genotype) or being too long and fail to do so (*MAOA-L* genotype). We suggest, that the other polymorphisms we found, which are not yet described in the literature (2.2R, 2.5R, 5.5R), are most likely too short or too long to act as an activator leading to a reduced expression of *MAOA* (MAOA-L).

7.2 MAOA genotype and its influence on partner choice

The MAOA-uVNTR genotype influences the expression level of the *MAOA* gene and furthermore the behavior of the individual (Buckholtz et al., 2008). In human populations, most cultures developed a monogamous mating system because human children need substantial paternal care (Lovejoy, 1981; Marlowe, 2000). Furthermore, in some cultures human females choose male partner with similar altruistic behavior

(Tognetti et al., 2014). Therefore our expectations were that the MAOA-uVNTR genotype would influence the human partner choice. Women would rather choose a partner with similar character traits (McCrae et al., 2008) and similar social behavior. We suggested that this would also apply to the *MAOA* genotype. Women with a certain MAOA-uVNTR genotype (*MAOA-L* or *MAOA-H*) would more likely choose a partner with a matching MAOA-uVNTR genotype (assortative mate choice). In our experiments we could not find any significant relationship between the MAOA-uVNTR and the partner choice in 28 married couples. One reason for this might be that the *MAOA* genotype mainly influences the behavior of males and not the behavior of females. This is because of the fact that the epigenetic modification of the *MAOA* gene influencing its expression plays an important role in woman, but apparently not in men (Wong et al., 2010). Another reason might be that the activity of *MAOA* is directly regulated by estrogen, a sex-specific hormone. Estrogen receptors are expressed in amygdala and orbitofrontal cortex (MacLusky et al., 1986) where they regulate the transcription of *MAOA* (Gundlah et al., 2002). Some studies showed that the *MAOA-L* allele has little to no effect on female brain development and behavior (Chakravorty and Halbreich, 1997; MacLusky et al., 1986; Gundlah et al., 2002; Ou et al., 2006). In males however, testosterone may influence the transcription of *MAOA* by acting through glucocorticoid/androgen response elements in the *MAOA* promoter (Ou et al., 2006) and *MAOA-L* can effect brain development (Buckholtz et al., 2008). The fact that the *MOAO* gene is located on the X chromosome should also be considered. Due to of the location on a sex chromosome the male offspring can inherit the gene only from the mother. Furthermore, only female offspring (*MAOA* allele has little to no effect on female brain development and behavior (Chakravorty and Halbreich, 1997; MacLusky et al., 1986; Gundlah et al., 2002; Ou et al., 2006) can inherit the second copy of this X-linked gene from the father. Thus, only the female *MAOA* genotype will affect the *MAOA* phenotype of the male offspring. Furthermore, in female offspring one of their X chromosomes copies will randomly be inactivated during the two-cell or four-cell stage of the embryos and will not code for any proteins (Cheng MK and Disteche CM, 2004). Based on our results and the sex dependent effect of *MAOA* we suggest that there is no correlation between the *MAOA* genotype and mate choice in humans.

7.3 HLA genotype and its influence on partner choice

Many studies suggest that the HLA genotype influences the partner choice in humans. A partner with a dissimilar HLA genotype to their own is preferred to get heterozygote offspring (Havlicek & Roberts, 2009). There are also studies with mixed results. They propose that the MHC genotype affect the mate choice in some but not all populations (Khankhanian et al., 2010; Ober, 1999; Derti et al., 2010). Some studies also suggest that there is no relationship between the HLA gene complex and the human mate choice (Hill et al., 1991). However, HLA-heterozygosity is associated with an advantage in the adaptive immune response (Carrington et al., 1999). Studies also showed that a high HLA diversity in the potential partner is also preferred (Lie et al., 2008). In the present study we only tested if the HLA alleles of 51 married couples are more or less dissimilar than the average dissimilarity across all of the test persons in the study. There was no significant difference in the HLA-A, HLA-DR1 and overall similarity in the married couples compared to the HLA similarity across all test persons. However, there was a significant difference in the similarity in HLA-B of married couples compared to HLA-B similarity across all test persons Thus, no MHC-disassortative mate choice could be observed in HLA-A or HLA-DR1, but for the HLA-B gene. The HLA-B allele is the most diverse gene across all HLA class I genes and across the whole HLA complex with more than 4828 different alleles (http://www.ebi.ac.uk/ipd/imgt/hla/stats.html). It plays a major role in antigen presentation and some HLA-B alleles (HLA-B27 and HLA-B57) are associated for example with slowing down the progress of HIV infections (Carrington et al., 2003). The HLA-B53 allele is most common in the West African population and has been shown to help against malaria (Hill et al., 1991). In birds the MHC class I (mainly MHC-B, the equivalent to HLA-B in humans) is crucial for parasitic resistance (Ekblom et al., 2004). Furthermore, different HLA alleles have also been found in genome-wide association studies (GWAS) associated with various autoimmune diseases such as systemic lupus erythematous, rheumatology and type 1 diabetes. Thus, HLA plays a key role in immunity not only in immune defense, but also proper discrimination from self- and non-self proteins. Moreover, studies also showed that a matching for HLA-B alleles in couples (same allele) is associated with fetal loss (Ober et al., 1998; Ober 1999). On the other side, HLA-B dissimilarity in couples is linked

with enhanced sexual satisfaction of both partners and the wish to have children (Kromer et al., 2016). HLA-B allelic diversity has already been found in previous studies to be associated with disassortative mate choice (Kromer et al., 2016). The odour attractiveness of HLA-B heterozygous males was also rated higher than of HLA-B homozygous males (Thornhill et al., 2003). MHC class I genes in general (especially HLA-B) have a greater impact on sexual satisfaction and the wish for children in human couples than MHC class II (Kromer et al., 2016). Similar observations for the preference of the odour of MHC dissimilarity in the partner choice were made in mice (Yamazaki et al., 1976). However, in humans the cultural factor may also influence the effect of the HLA allele in partner choice (Hedrick et al., 1997) given the fact that all couples were from the same population (Switzerland, all individuals with European genetic ancestry). Furthermore, we only tested 51 married couples in our study. A study including the HLA data from more couples from a wide variety of populations would support or contrast our result. Despite the small sample size, our results and the results obtained by other studies (Carrington et al., 1999; Penn, 2002; Wedekind et al., 1997) support the hypothesis that mate choice in humans is at least to some degree influenced by the MHC complex.

7.4 LD between SNP rs5953210 and Monoamine oxidase A-uVNTR

We were also interested if there is a correlation between the observed SNP rs5953210 and the number of tandem repeats in the MAOA-uVNTR. LD between an SNP and the MAOA-uVNTR would simplify tests to determine the *MAOA* expression level which is influenced by the number of repeats in the VNTR. Other studies already showed that there is a correlation between different SNPs in the *MAOA* gene and expression level (Pinsonneault et al., 2006). In this study an X^2 test was done to test for a LD. The deviation (D) in the observed frequency of haplotypes from the expected frequency was calculated. We showed, that there is indeed a LD between the SNP rs5953210 and the number of repeats in the MAOA-uVNTR. A “G” in the SNP is linked to 3.5 repeats in the MAOA-uVNTR leading to the *MAOA-H* genotype and an “A” in the SNP is linked to 4.5 repeats in the MAOA-uVNTR leading to a *MAOA-L* genotype. As described in “7.1 Number of repeats in the MAOA u-VNTR”, we currently cannot explain which impact the 4.5R has on the expression level.

These results are of great importance for further experiments to check the *MAOA* expression level only by observing single SNPs, which is easier and cheaper than counting the number of repeats in the MAOA-uVNTR.

8. Conclusion

This study showed that HLA-B plays a role in pair bonding in humans. We confirmed previous studies which suggest that HLA (or MHC) disassortative mating preferences drives genetic diversity in this gene complex. In contrast we did not find any evidence for the influence of *MAOA* genetic polymorphism on mate choice preferences in humans. An interesting question appears: Why does it seem that mainly the HLA-B gene influences mate choice? HLA-B also plays an important role in partnerships by enhancing sexual satisfaction of both partners and affecting the wish for child and fetal loss. It seems that HLA-B not only “helps” to find a matching partner but also enhances the quality of a relationship in fitting couples. A possible explanation might be the highest diversity of HLA-B (over 4828 alleles) among all HLA genes and consequently the big impact on the ability of the immune system to recognize a wide variety of antigens. It should also be considered that the HLA-A and HLA-C gene are very diverse (HLA-A: 3968 alleles, HLA-C: 3579 alleles).
On the other hand, *MAOA* genetic polymorphism does not seem to have any impact on mate choice despite its role in social behavior and aggression. Similar tests should be done with other genes influencing social behavior to figure out how strong the impact of such genes on mate preference in humans is in total. For me it seems very likely that matching personality and behavior do play an important role in social high developed animals/living beings like humans.

9. List of figures

10. List of tables

11. Literature

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