Assessing the association between cytokine-related genes and facial appearance

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Preface

Extensive research has been directed towards assessing the belief that facial appearance indicates health, particularly immunity. Many studies have linked facial appearance in the non-clinical populations with various health parameters such as self-reported health, cardiovascular health and even direct measures immunity such as response to hepatitis B vaccinations and cytokine response. There has also been direct evidence that genes associated with the immune response influence facial appearance in men. Being a genetics student I was particularly curious in the genetic link between facial appearance and health. The HLA genes are the only genes that have been investigated to determine this link and they represent a relatively small portion of the immune system. Cytokine genes were chosen as candidates for this dissertation to understand how immune based genes may influence facial appearance. Cytokines are good candidates as they play a vital role from start to finish in the entire immune response as well as general immune homeostasis.

This dissertation comprises of three chapters. **Chapter one** is a brief overview of the literature. Particular interest was given to (a) the importance of facial appearance in everyday life and in various fields of research, (b) to previous studies that assessed the genetic variants which influence facial appearance in non-clinical populations and (c) specific relationships between facial appearance and health. The general immune response was also investigated and particular focus was given to cytokines and the specific candidate cytokines that were used in this study. **Chapter two** is the research chapter in which I investigated the relationship between candidate cytokine-related SNPs and facial appearance. The chapter was divided into two experiments with each experiment assessing different facial appearance variables. In experiment one I determined the health appearance of facial images and the facial cues that influence health appearance. In experiment two I measured structural components of the face. In **Chapter three** I summarised the results and importance of this study, acknowledged the challenges and limitations as well as indicate suggestions for future studies.

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List of Figures

pg.

List of Tables

pg.

List of Abbreviations

BMI:	Body mass index
CSF:	Colony-stimulating factor
FA:	Fluctuating assymetry
HLA:	Human leukocyte antigen
ICHH:	Immunocompetence handicap hypothesis
IFN:	Interferon
IL:	Interleukin
MAF:	Minor allele frequency
ms:	milliseconds
PC:	Principal component
PCA:	Principal component analysis
ROS:	Reactive oxygen species
SD:	Standard deviation

Contents

Chapter 1

Literature review

1.1. The Importance of Facial Appearance

1.1.1 The role of facial appearance in sociology and psychology

Facial Appearance plays a crucial role in our everyday lives. As humans, we have developed a very complex way of recognising and extracting information from faces (Adolphs *et al.* 1998; Said *et al.* 2011); an imperative skill as the face is a complex trait that revels a wealth of information about an individual. Although we are constantly told "don't judge a book by its cover", intentionally or unintentionally; we form opinions and make predictions of people based on their appearance within seconds of meeting them (Willis and Todorov 2006). We infer intelligence (Zebrowitz *et al.* 2002; Talamas *et al.* 2016), personality (Hassin and Trope 2000; Little *et al.* 2006) and even the general health of a person based on facial appearance (Henderson *et al.* 2016). However, how accurate are we in judging individuals solely based on their faces? An important question, as our judgements based on facial appearance play an important role in who we choose to employ (Little and Roberts 2012), vote for (Little *et al.* 2007a), associate with, as well as who we choose to marry and have children with (Rhodes 2006; Little *et al.* 2011).

Previous studies showed that people can accurately predict the big five personality traits (openness to experience, neuroticism, agreeableness, conscientiousness and extraversion) from faces (Penton-Voak *et al.* 2006; Little and Perrett 2007b; Kramer and Ward 2010). In addition extraversion can even be accurately perceived after just 50 ms of exposure to a face (Borkenau *et al.* 2009). Cognitive abilities such as intelligence (Penton-Voak *et al.* 2006; Kleisner *et al.* 2014), physical traits such as strength (Sell *et al.* 2009), height (Schneider *et al.* 2013) and weight (Coetzee *et al.* 2009; Schneider *et al.* 2013) as well as biological traits such as health (Kalick *et al.* 1998; Zebrowitz and Rhodes 2004; Kramer and Ward 2010) and fertility (Penton-Voak and Chen 2004; Smith *et al.* 2006) have also been shown to be predicted from faces with an accuracy above chance. Even attractiveness, a trait that is commonly thought to be subjective, is judged accurately and reliably in faces; as there is an agreement in attractiveness ratings across gender, cultures and ages (Slater *et al.* 1998; Langlois *et al.* 2000; Zebrowitz *et al.* 2011; Coetzee *et al.* 2014).

1.1.2 The role of facial appearance in evolutionary studies

Facial appearance is not just important in sociology and psychology but important findings have also improved our understanding of anthropology and human evolution (Little *et al.* 2011). As mentioned earlier, there is an agreement across individuals and even cultures of what is found attractive (Langlois *et al.* 2000), even from infancy (Samuels *et al.* 1994). Findings that reveal facial attractiveness is not random has led to further research as to why certain facial features are considered attractive. The “good-genes” hypothesis provides a possible explanation as to why certain traits are found attractive and why we seek out these attractive traits in potential partners. According to the “good-genes” hypothesis, the females of a species seek out males that exhibit traits which could indicate an ability to pass on genetic variation to her offspring that would enhance their survival and reproductive success (Hamilton and Zuk 1982). Physical traits such as size, shape, symmetry and brightness of colours in fur, feathers or fins are selected for because they indicate genetic fitness, fertility and health (Andersson 1982; Andersson 1994; Moller and Thornhill 1998). Similarly, facial attractiveness in humans influences who we choose as possible mates and future parents of our children (Thornhill and Gangestad 1999).

Particular facial traits in humans could therefore be found attractive and influence mate choice because they could indicate the health and fertility of the individual. There are certain facial traits that have been extensively studied, that influence attractiveness and are found to be associated with both perceived and actual health. These traits are facial symmetry (Rhodes *et al.* 1998; Jones *et al.* 2001; Zaidel *et al.* 2005; Thornhill and Gangestad 2006; Gangestad *et al.* 2010), facial adiposity (level of fatness; Coetzee *et al.* 2009; Coetzee *et al.* 2011; Tinlin *et al.* 2013; Foo *et al.* 2017a), sexual dimorphism (masculinity/femininity; Perrett *et al.* 1998; Little *et al.* 2002; Smith *et al.* 2009; Rantala *et al.* 2013a), closer resemblance to the population average (Langlois and Roggman 1990; Rhodes *et al.* 2001) and skin homogeneity (Fink *et al.* 2006) and colour (specifically light, red and yellow skin tones; Stephen *et al.* 2009a; Stephen *et al.* 2011; Carrito *et al.* 2016). The relationship between these facial traits and health will be discussed in more detail in section 1.3.

1.1.3 The role of facial appearance in the clinical field

Recent efforts have been dedicated towards the use of facial appearance as a tool in the medical field. Physicians generally use visual cues, a method known as the “gestalt method”, for the diagnosis of a variety of diseases, especially diseases with non-specific signs or symptoms such as diagnosis of a pulmonary embolism (Lucassen *et al.* 2011). Many genetic disorders and syndromes, such as Down syndrome and Noonan syndrome, have characteristic facial features that are also used to aid in diagnosis; some examples are included in Table 1.1. There is however disagreement between physicians on the gestalt method, especially in faces that are not Caucasian (Lumaka *et al.* 2017). Improved facial imaging would provide physicians with further information that is more objective to help guide them in making a more confident diagnosis that would relieve diagnostic conflict. Improved imaging techniques will also ensure more accurate diagnosis in populations that are not commonly studied (e.g. Africans) as facial indicators for clinical diagnosis do differ between populations (Kruszka *et al.* 2017). Accurate visual diagnosis could even aid in underdeveloped, poorer regions in the world, where laboratory tests may not be readily available or affordable.

In recent years, the development of 3D geometric morphometrics (the statistical analysis of face shape) allowed researchers to identify very subtle facial dysmorphologies (Hammond 2007). This technology can improve diagnosis for the syndromes like those mentioned in Table 1.1, especially in cases where phenotypes may present differently with varying degrees of severity. For example, 3D geometric morphometrics can identify individuals with Noonan syndrome and velo-cardial-facial syndrome with 95% accuracy (Hammond *et al.* 2004), Down syndrome with an accuracy of 81-99% (Ferry *et al.* 2014) and foetal alcohol syndrome (FAS) with 94% accuracy (Mutsvangwa *et al.* 2010). More sensitive diagnostic techniques can even detect morphological differences between certain mental disorders, such as schizophrenia (Hennessy *et al.* 2007), bipolar disease (Hennessy *et al.* 2010) and autism spectrum disorder (Hammond *et al.* 2008) compared to their sex and aged matched controls.

Table 1.1. *Genetic disorders that are often characterised according specific facial features. Information was gathered from Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org)*

Disorder	Examples of characteristic facial features	Genetic abnormalities that could be involved in facial appearance	OMIM entry
Clift lip/palate	Split in the upper lip and/or roof of the mouth	Mutations in *PVRL1*	MIM #225060
Down syndrome	Upward-slanting eyes, folds on the inner corner of the eyes and a flat nasal bridge	Trisomy 21	MIM #190685
Williams-Beuren Syndrome	Flat nasal bridge, short upturned nose, eye puffiness, long philtrum, and broad forehead and mouth in young children. Slight coarse features, with full lips, a wide smile, and a full nasal tip in older patients	Deletion of *GTF2IRD1*	MIM #194050
Prader-Willi Syndrome	Almond-shaped eyes, narrowing of the forehead at the temple, narrow bridge of the nose, thin upper lip and a downturned mouth	Loss of function in a region of chromosome 15	MIM #176270
Treacher Collins Syndrome	Downward slanting eyes, underdeveloped or absence of cheekbones and/or brow ridge and a small or slanting lower jaw	Mutations in *TCOF1*, *POLR1C* and *POLR1D*	MIM #154500
DiGeorge Syndrome	Puffy and upward slanting eyes, elongated nose with a large tip, and a small mouth with an outward turned top lip	Mutations in *TBX1*	MIM #188400
Kabuki syndrome	Abnormally long opening of eyelids, lower eyelids are turned outward, arched eyebrows, and a broad nose with a flattened tip	Mutations in *KMT2D* or the *KDM6A*	MIM#147920 and MIM#30869
Roberts syndrome	Cleft lip/palate, wide set, downward slanting eyes, a beak shaped nose with small nostrils and possible presence microcephaly	Mutations in *ESCO2*	MIM #268300

1.1.4 The role of facial imaging in forensics

Interest has recently grown in terms of the genetics that influence facial appearance. DNA left at a crime scene can be used to determine the suspect's or an unknown victim's external visual characteristics such as hair type and colour, eye colour, skin pigmentation, ancestry and general facial shape (Kayser and De Knijff 2011; Kayser 2015). Recent preliminary studies have now been able to predict more information of an individual's facial appearance and morphology based solely on genetic information (Peng *et al.* 2013; Claes *et al.* 2014b). However these preliminary studies have centred on admixed (African/European) or Eurasian populations, no studies have been performed in a homogenous African population (Claes *et al.* 2014a; Qiao *et al.* 2016). African faces are morphologically different from Caucasian, Asian and even admixed populations (Sporer 2001; Farkas *et al.* 2005; Kau *et al.* 2010; Talbert *et al.* 2014). Further investigation for 3D facial morphological prediction in African faces as well as gathering a larger database of genes that influence facial appearance is important to further expand the field of facial morphology genetics so that one day it could be applied in real-life forensic situations.

1.2. The Genetics of Facial Appearance

1.2.1 Genetic influence in the face

It is clear that studying the genetics of facial appearance is highly beneficial and there is still substantial information that is yet to be discovered. The face is a product of both environmental and genetic factors and their interaction with each other. Although the environment does play an important role, the face is highly heritable (Djordjevic *et al.* 2016) with narrow-sense hereditability estimates being as high as 0.8 (Johannsdottir *et al.* 2005; Weinberg *et al.* 2013; Cole *et al.* 2017). The genetic influence in facial appearance is evident when observing the facial resemblances between twins and within families (Johannsdottir *et al.* 2005; Weinberg *et al.* 2013). Facial similarities are also seen amongst people from the same ethnicities and geographical populations (Farkas *et al.* 2005). Finally, further proof of genetic influence of the face is found in the clinical populations, as observed in Table 1.1. Genetic abnormalities can lead to shared, characteristic facial dysmorphologies, implying the genetic abnormality is involved in facial morphology. There are a number of complex pathways involving many genes and enhancers that are continuously switched on and off, and this eventually leads to the development of the face

(for a review see (Francis-West *et al.* 1998)). However, surprisingly little is known about the genes that influence the variation we observe in facial appearance. Most studies that aim to assess the genes involved in facial appearance involve the clinical population where facial development has been disrupted (e.g. Table 1.1; Wilkie and Morriss-Kay 2001; Suri 2005; Hochheiser *et al.* 2011).

1.2.2 Candidate gene studies

Recently, a number of studies have been conducted in non-clinical populations to assess the genetic influence on facial appearance. Boehringer *et al.* (2011) used a candidate gene approach to assess the genes influencing facial morphology in participants who suffer from non-syndromic cleft lip/palates. Those genes were then assessed for the impact that they have on normal variation in the non-clinical population. They found an association between SNP rs1258763, near the *GREM1* gene, and nose width in 2D facial images of a German cohort (Boehringer *et al.* 2011). An association was also found between SNP rs987525, near the *CCDC26* gene, and bizygomatic distance (distance between the two cheek bones) in 3D facial images of a Dutch cohort (Boehringer *et al.* 2011). A few other candidate gene studies have been performed to assess their associations with facial appearance and morphology (Peng *et al.* 2013; Claes *et al.* 2014b; Peng *et al.* 2016). Peng *et al.* (2013) assessed the associations between 3D facial images and 10 candidate SNPs across four genes in a Han Chinese population. Their most significant finding was an association found between a SNP in *IRF6* and lip shape. A similar study used an admixed African/European population to model facial morphology using the effects of 68 ancestry informative markers, a sex marker and 24 SNPs across 20 genes. They found significant associations with facial appearance across 20 of the genes (Claes *et al.* 2014b). The association between an *EDARV* gene with 16 facial characteristics was investigated in a young adult Turkey/Eastern-Asian population; significant associations were most notably found within the chin region (Peng *et al.* 2016).

1.2.3 Genome-wide association studies

The advancement of gene sequencing technologies has led to a large increase in genome-wide association studies (GWAS). The first GWAS studies examining the genetics that influence facial appearance in a non-clinical population was in 2012 (Liu *et*

al. 2012; Paternoster *et al.* 2012). Paternoster *et al.* (2012) and Lui *et al.* (2012) both found an association between *PAX3* and nasion position in a GWAS study performed and replicated in large European cohorts; Paternoster *et al.* (2012) used only 15 year olds and Lui *et al.* (2012) used a large age range between 12- 60 years. Lui *et al*. (2012) also found significant associations between SNPs in 4 other genes (*PRDM16, TP63, C5orf50* and *COL17A1*) and facial appearance (significant findings were mainly observed in the nasion position and distance between the eyes).

A surge of GWAS studies for facial appearance was again observed in 2016 (Adhikari *et al.* 2016; Cole *et al.* 2016; Qiao *et al.* 2016; Shaffer *et al.* 2016). A GWAS conducted in Eurasian and Hans Chinese cohorts revealed SNPs in the *UBASH3B, COL23A1* and *PCDH7* genes and a SNP at a position on the p arm of chromosome 2 were significantly associated with the distance between external and internal corners of the eyes, nasal shape, mouth shape and cheeks respectively (Qiao *et al.* 2016). Shaffer *et al.* (2016) investigated the relationship between 20 quantitative facial measurements in a large European sample (aged 3-49) and just under a million genotyped SNPs. They found significant associations with cranial base length and the *MAFB* and *PAX9* SNPs, the distance between the corners of the eyes and *ALX3* and *HDAC8* SNPs, nasal shape and two SNPs found in *PAX1* and the q arm of chromosome 14 and finally, upper facial depth and a SNP on the q arm of chromosome 11 (Shaffer *et al.* 2016). Like Peng *et al.* (2016), Adhikari *et al*. (2016) also found an association between an *EDARV* gene and chin protrusion in a GWAS study performed in a large Latin American sample. They also found significant associations with nose shape and the SNPs in *DCHS2, RUNX2, GLI3* and *PAX1* genes (Adhikari *et al.* 2016). Cole *et al.* (2016) is the first study that used a homogenous Bantu speaking African sample. The GWAS was conducted in Tanzanian children and adolescents. Significant findings that were novel which included SNPs within the *SCHIP1* and *PDE8A* genes that were associated with facial size. Studies assessing the genes that drive facial appearance have yet to be performed in an adult African population.

1.3. The Relationship between Facial Appearance and Health

Our faces reveal extensive information about ourselves, including our health. It's already been mentioned that our faces can indicate genetic quality (Lie *et al.* 2010) and both mental (Hennessy *et al.* 2007; Kleisner *et al.* 2014), and emotional (Hennessy *et al.* 2010)

health. Extensive research has also been conducted into how our faces portray biological health (Kalick *et al.* 1998; Kramer and Ward 2010; Henderson *et al.* 2016). Attractiveness and perceived health of an individual are highly positively correlated (Hume and Montgomerie 2001; Jones *et al.* 2001; Rhodes *et al.* 2007; Foo *et al.* 2017a; Phalane *et al.* 2017) . Certain characteristic facial features are found to be attractive because they could potentially indicate a healthy partner that can provide both direct and indirect benefits (Hamilton and Zuk 1982; Thornhill and Gangestad 1999). Direct benefits include a healthy immune system; meaning they will likely be sick less often, have a longer life-span and provide better parental care and resources (Møller and Jennions 2001; Kokko *et al.* 2003). Indirect benefits include the ability to pass on "good-genes" to many offspring that can thrive into the next-generation (Kirkpatrick and Ryan 1991; Andersson 1994). Extensive research has been conducted into several attractive and healthy-looking facial cues that could possibly indicate actual biological health (Thornhill and Gangestad 1999; Fink and Penton-Voak 2002; Phalane *et al.* 2017). The facial cues that will be further discussed include symmetry, averageness, sexual dimorphism, adiposity and skin colour (Matts *et al.* 2007; Rhodes *et al.* 2007; Coetzee *et al.* 2009).

1.3.1 Facial symmetry

The various aversive conditions that individuals are exposed to throughout their lives may impact their physical development (Schell *et al.* 2009). This may lead to small and random deviations in features that are commonly symmetrical, such as the face (Møller and Thornhill 1997; Thornhill and Møller 2007). Deviations from bilaterally symmetrical features are often described as fluctuating asymmetry (FA). It is thought that FA, is the type of asymmetry in the face that is associated with developmental instability (Van Valen 1962; Leary and Allendorf 1989; Gangestad *et al.* 1994). Examples of aversive conditions that could cause FA include genetic abnormalities (that may arise via mutations and inbreeding) and environmental stressors (such as disease or lack of recourses; Møller and Thornhill 1997; Møller and Swaddle 1997; Kowner 2001). Therefore, it is hypothesised that symmetry in the face signals health as it could indicate better genetics and an ability to overcome stressful environmental conditions (Thornhill and Gangestad 1993; Møller and Thornhill 1997; Thornhill and Gangestad 1999). Symmetry or asymmetry in the face is detected in a variety of ways. Firstly, it can be physically measured as distances between landmarks on a facial image (Shackelford and Larsen 1997; Jones *et al.* 2001). Secondly,

a mirror image of half a facial image is created and the face is then adjusted along an axis from perfectly symmetrical to very asymmetrical (Rhodes *et al.* 2001; Rhodes *et al.* 2007). Finally, how symmetrical a face appears can be judged by participants (Zaidel *et al.* 2005; Phalane *et al.* 2017). Regardless of whether facial symmetry is measured or judged, it is consistently perceived as healthier (Jones *et al.* 2001; Rhodes *et al.* 2001; Zaidel *et al.* 2005; Rhodes *et al.* 2007).

Shackelford and Larson (1997) was one of the first studies to positively link FA to poorer health; measured as self-reported psychological, emotional and physiological measures. Some self-reported general health measures such as trouble concentrating, lower activity levels, trouble sleeping, muscle soreness etc. were found to be positively correlated to asymmetry. However, other measurements such as headaches runny noses and sore throats were not significant. General observations of the results from this study also revealed stronger relationships between symmetry and health in men compared to women (Shackelford and Larsen 1997). Thornhill and Gangestad (2006) found a significant positive correlation between self-reported number of respiratory infections (but not stomach or intestinal infections) and facial asymmetry in men, no significant relationships were found in women. A higher concentration of urinary biomarkers for oxidative stress were also found to be significantly correlated with FA in men (Gangestad *et al.* 2010). Oxidative stress is a biomarker of health used to measure oxidative damage caused by excess reactive oxygen species (ROS). Oxidative damage leads to mutations and other DNA damage which could result in cancers and neurodegenerative disorders (Cooke *et al.* 2003).

Not all studies found significant associations between symmetry and heath (Van Dongen and Gangestad 2011; Pound *et al.* 2014). Van Dongen and Gangestad (2011) performed a meta-analysis on nearly 100 studies that have assessed the relationship between overall FA and various measures of health and quality. These measures of health and quality include infectious diseases, congenital susceptibilities, foetal outcomes, psychological disorders, reproductive outcomes, attractiveness and hormonal effects. The meta-analysis revealed only a weak mean effect size (Pearson's r=0.2) and evidence of publication bias. When the publication bias was corrected for, the mean effect size decreased further (r=0.1). More recently, Pound *et al*. (2014) also failed to find an association between facial asymmetry in 3D facial images and longitudinal measures of childhood health. Moreover,

Phalane *et al.* (2017) failed to find a significant association between facial symmetry and cytokine response, a direct measure of immunity.

Roberts *et al.* (2005) aimed to assess a more direct relationship between facial symmetry and "good-genes" by assessing the heterozygosity in a set of genes described as the human leukocyte (HLA) genes. HLA genes are highly polymorphic genes that code for cell surface proteins that play an important role in antigen recognition in the immune response (Mungall *et al.* 2003). Heterozygosity in the HLA genes is thought to be desirable in offspring as it codes for a more diverse range of antigen proteins that can recognise a wider array of infectious diseases and therefore lead to an enhanced immunity (Penn *et al.* 2002). However, no significant association was found between facial symmetry and HLA heterozygosity (Roberts *et al.* 2005).

The relationship between facial symmetry and health could be more apparent in men than in women. However, most research that has been conducted to assess the relationship between symmetry and actual health have used self-reported measures of health. This could be the reason for inconsistent findings in the literature, as self-reported health has been suggested to be an undependable measure of health that is subjective (Rhodes *et al.* 2007; Phalane *et al.* 2017). More research that uses a direct measure of health or immunity should be conducted to confirm the link between facial symmetry and health.

1.3.2 Facial averageness

Similar to symmetry, facial averageness (how close the face resembles the population average) is also thought to be an indicator of an ability to overcome environmental adversities (Gangestad and Buss 1993; Møller and Swaddle 1997; Jokela 2009). Averageness may also indicate heterozygosity of genes and proteins, this may lead to a robust immune system that is able to recognise a more diverse range of antigens and therefore fight off a broader scope of pathogens (Thornhill and Gangestad 1993). Distinctive facial traits that deviate from the population average could also be a sign of genetic or chromosomal disorders, examples of extreme cases are presented in Table 1.1.

Certain software such as JPsycomorph allows for the merging of a number of faces into a single face (Tiddeman and Perrett 2001). As more faces merge into a single face, the

generated facial image reveals a closer resemblance to the population average. It is consistently found that the closer an individual's face resembles the population average, the healthier the face appears (Rhodes *et al.* 2001; Rhodes *et al.* 2007; Lie *et al.* 2008; Foo *et al.* 2017a).

The relationship between averageness and actual health has not been explored extensively however, Rhodes *et al*. (2001) has assessed the associations between both symmetry and averageness with actual health. In this study health was measured through a series of self-reported measures and medical records during childhood and adolescence (how often they were sick as children, antibiotic use etc.). They found that facial averageness was a reliable indicator of health in 17 year olds in both men and women, however no significant association was found between symmetry and actual health. It was also found that the relationship between averageness and actual health was more robust in people who were below the median in averageness (Zebrowitz and Rhodes 2004). Phalane *et al*. (2017) found no significant relationship between facial averageness and cytokine response. Although there is some evidence, more research will need to be conducted to assess if facial averageness is truly an indicator of actual health.

1.3.3 Sexual dimorphism in the face

Sexual dimorphism is a secondary sexual characteristic that refers to the level of masculinity in men and femininity in women. Secondary sexual characteristics arise due to an increase in hormone levels (testosterone in men and oestrogen and progesterone in women respectively) that starts during puberty. A masculine face is most commonly characterised as having more square and angular features; commonly observed in the jaw line. More masculine faces also tend to have thinner lips, narrow eyes and a prominent eyebrow ridge with larger eyebrows. A feminine face is characterised as having rounder features, plump lips, larger eyes and a smaller nose and jaw.

Sexual dimorphism is a facial cue that is hypothesised to indicate sexual and reproductive health as well as immune functioning (Perrett *et al.* 1998; Rhodes *et al.* 2003). Sex hormones have been positively linked to measures of fertility (Stewart *et al.* 1993; Lipson and Ellison 1996; Meeker *et al.* 2007). Therefore facial features that could indicate the hormone levels within the body, may also reflect reproductive health (Penton-Voak and

Chen 2004; Smith *et al.* 2006). Sexual dimorphism could also be an honest signal to immune functioning. The immunocompetent handicap hypothesis (ICHH) states that phenotypically masculine features presented in males are considered as honest indicators of health and genetic quality (Folstad and Karter 1992). This is because androgens such as testosterone act as an immunosuppressant (Grossman 1985). Therefore, only males that have superior immune systems can afford to have higher testosterone levels (which presents phenotypically as masculinity). The female hormone oestrogen is also considered an immunosuppressant, therefore the same immunocompetent handicap could also apply to femininity in female faces (Rhodes *et al.* 2003).

Studies have confirmed that perceived femininity in females is significantly positively correlated with oestrogen levels (Smith *et al.* 2006). Similarly, it's been found that men who are judged as more masculine, have higher levels of testosterone in their saliva (Penton-Voak and Chen 2004). Masculinity has also been linked to semen quality; determined through measures of rapid progressive motility, linearity of sperm movement, sperm concentration and percentage of motile sperm (Foo *et al.* 2017a). However, results have been conflicting (Peters *et al.* 2008). Peters *et al*. (2008) found no significant correlations between semen quality parameters (sperm concentration, motility and morphology) and judged facial traits; attractiveness, masculinity, averageness and symmetry.

Masculine and feminine looking faces in men and women respectively are indeed positively correlated to how healthy a person looks (Rhodes *et al.* 2003; Zebrowitz and Rhodes 2004; Rhodes *et al.* 2007; Scott *et al.* 2008; Smith *et al.* 2009). However, a few studies have reported that no significant association was found between masculinity and a healthier appearance (Boothroyd *et al.* 2005; Pentonvoak *et al.* 2007). Cultural effects have shown to influence the attractiveness of facial masculinity, as masculine features are found more attractive in areas that have lower standards of health (Penton-Voak *et al*. 2004; Scott *et al*. 2008; DeBruine *et al*. 2010). Therefore, culture and socio-economic status may also influence the relationship between masculinity and health perception. However, to my knowledge, no studies have investigated the effect that culture may have on the relationship between facial masculinity and health perception. Investigation into the relationship between facial masculinity and actual health has revealed more consistent results that provide support for the ICHH. Facial masculinity has been negatively

correlated with self-reported measures in the number and duration of colds and flu and antibiotic use. But no significant association was found between male masculinity and self-reported stomach illness (Thornhill and Gangestad 2006). Boothroyd *et al*. (2013) found similar results between masculinity and reported colds and flu infections. Rhodes *et al*. (2003) found that masculinity in men was only modestly associated with self-reported health measures in adolescents, the same study did not find any relationship between femininity and self-reported health in females. A more reliable indicator of health would use objective measurements of immune functioning rather than relying on participants' self-reported health measurements and medical records. Oxidative stress levels were found to be negatively correlated with masculinity in men (Gangestad *et al.* 2010). Masculinity was also significantly associated with a better immune response after a hepatitis B vaccination (Rantala *et al.* 2013a) as well as an enhanced cytokine response (Phalane *et al.* 2017). Lie et al (2008) however, found no association between masculinity and HLA heterozygosity (Lie *et al.* 2008).

The application of the ICHH in women is less clear. The relationship between oestrogen and immunocompetence seems to be weaker (Alexander and Stimson 1988). A possible explanation could be that feminine facial cues do not differ from immature traits compared to masculine features; therefore feminine traits may be less costly (Rhodes *et al.* 2003). A few studies have assessed the direct relationship between femininity in females and actual health. Rhodes *et al*. (2003) found no association between self-reported health measures and femininity (Rhodes *et al.* 2003). It was however found that less masculine faces in women were significantly correlated with less self-reported respiratory infections but no associations were found for antibiotic use or stomach and intestinal illness (Thornhill and Gangestad 2006). Gray and Boothroyd (2012) found partial evidence for the association that femininity has with self-reported illness; femininity was again associated with respiratory illness but not significantly with stomach illness. No studies have assessed the relationship between facial femininity and more direct measures of health. Overall, the relationship health and femininity in females is less clear than the relationship between health and masculinity in men. Masculinity and femininity may also only indicate certain measures of health (respiratory infections) but not others (immune response to stomach infections).

1.3.4 Facial adiposity

Weight is an important health indicator. Being underweight could indicate a number of illnesses or malnutrition that leads to low immune functioning (Ritz and Gardner 2006). Likewise, being overweight is highly significantly correlated with many health risks such as cardiovascular health, diabetes mellitus as well as inflammatory diseases and immune dysfunction (Visser *et al.* 1999; Kopelman 2007). Judgements on the fat levels within the face (facial adiposity) is a reliable indicator of overall weight and weight distribution; even a more reliable indicator than body shape, body mass index (BMI) or percentage body fat (Coetzee *et al.* 2009; Tinlin *et al.* 2013). For example, visceral fat is detrimental fat that is found around the abdomen and the amount of fat in the cheeks is a good indicator of the level of visceral fat in the body (Levine *et al.* 1998). Adiposity levels in the face significantly influences the health appearance of the face; people that appear underweight or overweight are perceived as less healthy (Coetzee *et al.* 2009; Fisher *et al.* 2014; Foo *et al.* 2017b).

Perceived facial adiposity has been negatively linked to longevity (Reither *et al.* 2009). Coetzee *et al.* (2009) revealed a significant association between facial adiposity and cardiovascular health (measured as blood pressure) and reported health (frequency and duration of colds and flu as well as antibiotic use); with heavier appearing faces having lower health scores. Tinlin *et al*. (2013) found a negative association when assessing the relationship between adiposity and self-reported physical and psychological condition as well as serum progesterone levels. Facial adiposity has also been linked to direct measures of immune functioning (Rantala *et al.* 2013a; Phalane *et al.* 2017). Antibody response to a hepatitis B vaccination, was negatively correlated with adiposity levels in men (Rantala *et al.* 2013a). However, Phalane *et al*. (2017), found a significant positive relationship between adiposity and cytokine response in South African men. This apparent contradiction is likely because the South African cohort in the Phalane *et al.* (2017) study consisted of substantially more underweight participants (BMI < 18.5 kg/m^2) compared to previous studies. Therefore underweight men had lower health scores than those who had a normal weight (18.5 kg/m^2 < BMI > 25 kg/m^2). Compared to the previous facial cues, facial adiposity reveals more of a curvilinear instead of a linear relationship with health. An optimal adiposity level is considered healthiest, as weight increases or decreases from this optimum, so does health. Studies assessing adiposity should contain an even distribution

of underweight, normal weight and overweight individuals to better understand its relationship with health.

1.3.5 Facial skin colour

Facial skin colour and homogeneity is a facial trait that influences health appearance (Jones *et al.* 2004; Matts *et al.* 2007; Stephen *et al.* 2011; Fink *et al.* 2012; Tan *et al.* 2017). Skin colour is most commonly measured on light-dark, red-green and blue-yellow axes. A lighter skin tone could positively be correlated with vitamin D absorption (Jablonski and Chaplin 2000). A somewhat redder skin tone is considered to be an indicator of higher oxygenation levels and better circulation. Higher oxygenation levels advertise enhanced fitness levels, more efficient circulation and cardiovascular and respiratory health (Johnson 1998; Armstrong and Welsman 2001; Boushel *et al.* 2001). An enhanced yellow skin tone has been related to a diet that consists of higher levels of carotenoid pigments (Stephen *et al.* 2011; Whitehead *et al.* 2012). Carotenoids such as B-carotene, lycopene, lutein and zeaxanthin are consumed in our diet through intake of orange and yellow fruits and vegetables, tomatoes and green leafy vegetables (Johnson 2002; Stahl and Sies 2005). Carotenoids are natural antioxidants that function to combat ROS in the body which cause most notably cancers, and chronic diseases resulting from cardiovascular risks, inflammation and autoimmunity (Johnson 2002). It is important to note that the colour measurements which are discussed here are not extreme colour changes in lightness, redness or yellowness, such as those observed with jaundice etc. All colour changes of skin tone discussed here are within the normal range.

A lighter, redder and yellower skin tone appears healthier (Stephen *et al.* 2009b) and this is consistent across ethnicities (Stephen *et al.* 2011; Tan *et al.* 2017). Stephen *et al*. (2011) conducted a study using both Caucasian and Black South African populations. A lighter and yellower skin tone was preferred and perceived as healthier in both populations however, when maximising the health appearance, enhancing a yellower skin tone through carotenoid pigmentation was more important than adjusting the face based on melanin pigmentation (which influences how light or dark a face appears). The perception of health due to melanin colouration could be somewhat influenced by ethnicity/culture. Having darker skin with greater melanin production is considered beneficial in areas closer to the equator, which receive harsh amounts of UV rays; the melanin acts as a protective agent.

Less melanin is considered desirable in areas further away from the equator with less sun as it allows for greater absorption of vitamin D (Jablonski and Chaplin 2000). The extent of the cultural influence on the relationship between melanin production and health appearance has not been directly tested. Tan *et al.* (2017) revealed that Malaysian Chinese faces were considered healthier due to the change in skin colouration caused by the consumption of carotenoid-rich smoothies. They found there was an optimal carotenoid skin colouration in the faces that was considered healthy; oversaturation of carotenoid skin colouration was perceived as less healthy (Tan *et al.* 2017). Phalane *et al*. (2017) found that redness, yellowness and lightness in the skin were all very strongly positively correlated and South African men that had yellower, redder and lighter skin tones were considered to appear significantly healthier (Phalane *et al.* 2017). Foo *et al.* (2017) did not however find this association between skin colour and health perception or immune functioning in Caucasian faces (Foo *et al.* 2017a)

Not many studies have been conducted to assess the relationship between skin colour and actual measures of health. Roberts *et al*. (2005) has revealed that healthier skin appearance is positively correlated to heterozygosity in the HLA gene region in Caucasian men (Roberts *et al.* 2005). Phalane *et al*. (2017) assessed the direct link between skin colour and immunity, measured as cytokine activity, in South African men. They found significant associations between cytokine levels and increased skin yellowness and lightness, but not redness. A study conducted in Caucasian participants found that after injections that induce an acute immune response, the skin colour became lighter and redness and yellowness was reduced (Henderson *et al.* 2017).

1.3.6 The genetic link between facial appearance and health

As mentioned earlier, there have been a number of studies assessing the genetics of facial appearance however very few genetic studies have been conducted to establish the link between facial appearance and health. The only genes to date that have been assessed are the HLA genes that have been briefly mentioned. The heterozygosity of HLA genes has been found to be indicative of facial appearance in British and Australian men (Roberts *et al.* 2005; Lie *et al.* 2008) but not South African or Australian women (Coetzee *et al.* 2007; Lie *et al.* 2008; Lie *et al.* 2010). These studies provide further insight into the genetic associations between facial appearance and health, particularly in men. However

HLA's represent a very small portion of the immune system and more genetic components of the immune system will have to be assessed to further understand the genetic associations between facial appearance and health.

1.4. The Immune System

The immune system is the body's complex defence system against invading foreign, and potentially harmful, agents. It also functions to remove dead or damaged cells within the body (Delves and Roitt 2000). For the immune system to be effective, it must be able to recognise a wide variety of foreign material (bacteria, viruses, parasites and fungi etc.) from the body's own healthy tissue; recognition between cells occurs through antigens such as HLAs (Klein and Sato 2000). After recognition, the immune cells must be able to communicate with one another (often through cytokines (Mantovani and Dejana 1989)) to bring about the appropriate response to remove the foreign invader (Behm *et al.* 2012). And finally, once the infection has been removed or contained, the immune response must then be supressed (Parkin and Cohen 2001; Abbas *et al.* 2016). The immune system is divided into two main responses; the innate and adaptive immune response (Figure 1.1)

An individual's health relies on the proper functioning of the immune system that requires the activity of many cells, proteins and signalling molecules working together. The immune system is complex and requires the integration of many networks, pathways and feedback loops to carry out the appropriate immune response. Dysregulation in any of these components results in immune malfunction either through over- or under-activation. An over-active immune system leads to autoimmunity and allergies (Wilder 1995) whereas an under-active immune system leads to chronic illnesses, infections and even cancers (Cremer *et al.* 1990).

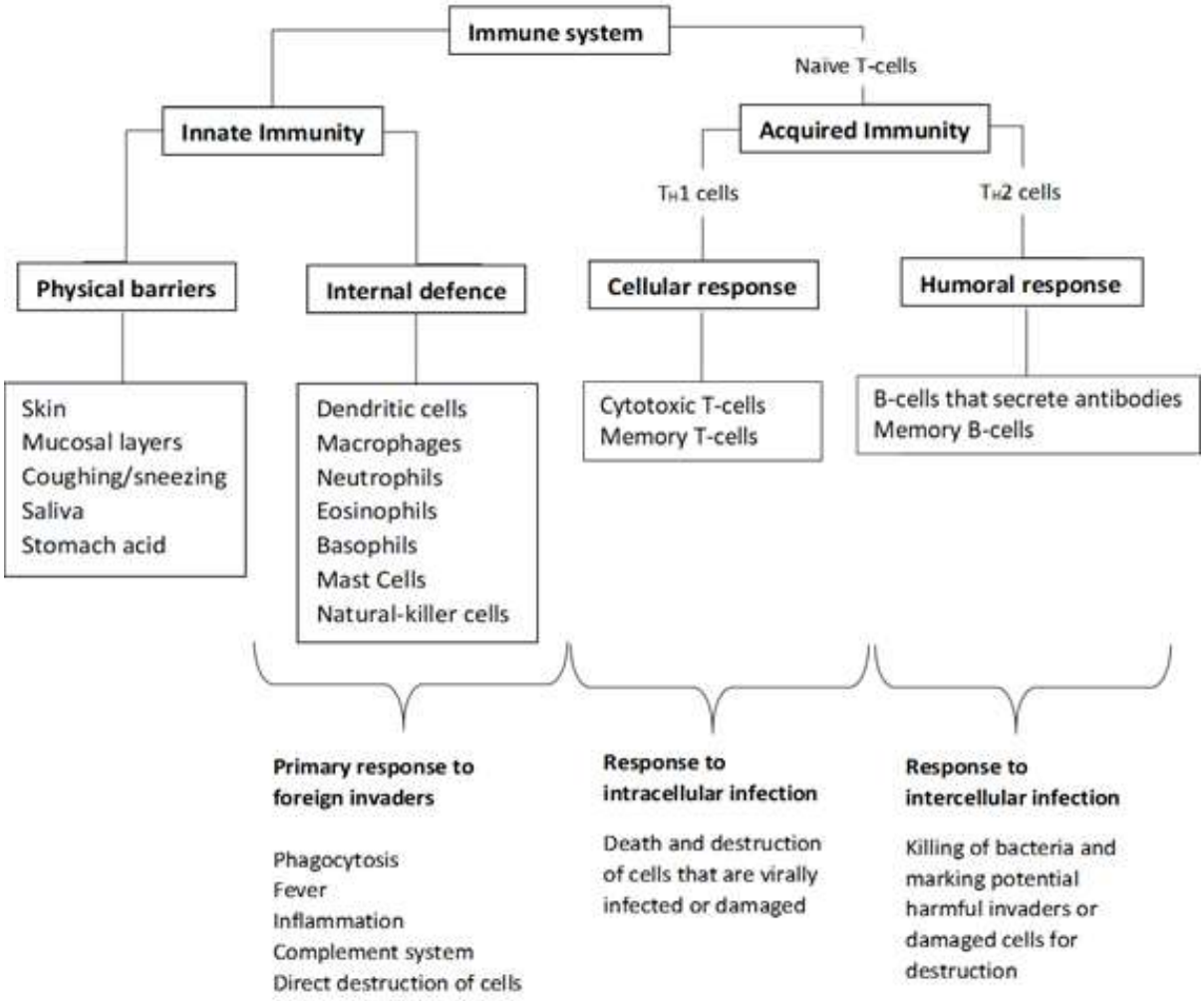


Figure 1.1. *Overview and division of the immune system responses and its components*

1.5. Cytokines

A very important component of the immune system that will be the centre of focus in this study are the cytokines. Cytokines are small proteins or glycoproteins that allow for communication between the immune system and other systems in the body such as the endocrine and nervous system (Besedovsky and Del Rey 1992; Ader *et al.* 1995). Cytokines also allow for cells within the immune system to communicate with one another; they govern and conduct the entire immune response as well as maintain homeostatic control through receptor binding and altering gene expression in target cells (Spurlock 1997; Vilček 2003). Cytokines can be autocrine (acts on the cell from which it is secreted from), paracrine (binds to receptors on neighbouring cells) and even endocrine (travels

through the bloodstream and binds to distant cells; Vilček 2003). Cytokines are a large group of proteins however seven cytokines were of particular interest in this study and will be discussed further, these include various interleukins (IL-2, IL-4, IL-6, IL-8, IL-10), colony stimulating factor 2 (CSF2) and interferon gamma (IFN- γ). These cytokines in particular were of interest as they play a role in both the innate (IL-6) and adaptive immunities (IL-8 and CSF2; Shi *et al.* 2006; Mantovani *et al.* 2011). As well as both the T_H1 immune response (IL-2 and IFN-γ), the T_H2 immune response (IL-4, IL-6 and IL-10; Romagnani 1999) pathways.

1.5.1 Interleukin-2

The interleukin- 2 gene is found on chromosome 4. *IL-2* was initially described as a T-cell growth factor that results in growth, differentiation and survival of T-lymphocytes (Morgan *et al.* 1976). It plays a role in the cellular immune response that is involved in intracellular infections and dysfunctions such as viruses and cancers (the T_H1 immune response pathway; Figure 1.1). *IL-2* does not only act in response to intracellular pathogens, but it plays a role in immune homeostatic function (Boyman and Sprent 2012). To understand the importance and function of *IL-2*, its role in disease and immune therapy has been assessed. In humans, the defective *IL-2* gene results in reduced T-cell response and immunodeficiency (Chatila *et al.* 1990). In immunotherapy, an anti- IL-2 receptor antibody (anti-Taq) is used to treat T-cell leukaemia and lymphomas and reduce allograft rejection and inflammation (Waldmann and O'shea 1998; Morris and Waldmann 2000). Alternatively, enhancement of the IL-2 response in humans is used to treat HIV and certain tumours (Atkins *et al.* 1999; Paiardini *et al.* 2001). Mice that have had their *IL-2* gene silenced also have reduced T-cell response; interestingly, autoimmunity, not immunodeficiency develops (Sadlack *et al.* 1993). This could be due to the fact that IL-2 also has inhibitory effects to downregulate the T-cell immune response through a negative feedback loop. IL-2 is therefore important in maintaining (both to inhibit and enhance) the T_H1 immune response to intracellular infections (Gaffen and Liu 2004). Although it's primarily involved in the cellular immune response, the cytokine also plays a role in the humoral immune response as it has been found to promote B-cell proliferation (Mingari *et al.* 1984).

1.5.2 Interleukin-4

This interleukin gene is found on chromosome 5 and is also important in the adaptive immune response. IL-4 however supresses the cellular response through inhibiting production of IFN-γ and IL-2 (Gautam *et al.* 1992) and enhances the humoral immune response pathway that reacts to intercellular pathogens (the T_H2 pathway; Swain *et al.* 1990; Figure 1.1). IL-4 leads to differentiation of T_H2 cells from naïve T-cells and causes activation and proliferation of B-cells, primarily resulting in IgE production (the immunoglobulins involved in allergies; Parronchi *et al.* 1992). The interleukins' role in the T_H2 immune response makes it very important in the allergic reaction and it could be used as a target to treat allergies (Racke *et al.* 1995; Borish *et al.* 2001). The cytokines inhibitory effect could also possibly be used as a treatment for conditions where the T_H1 response pathway is overactive; as is observed in chronic inflammatory diseases.

1.5.3 Interleukin-6

This interleukin is an important pro-inflammatory cytokine found on chromosome 7. It is primarily involved in the innate immune response but it also plays a role in the adaptive immune response. The primary function for IL-6 is to bring about inflammation (Fattori *et al.* 1994). This interleukin also interacts with the nervous system, specifically the hypothalamic-pituitary-adrenal axis, to produce a fever (Chai *et al.* 1996). Dysregulation in IL-6 production results in a variety of diseases. Abnormal levels of IL-6 are found in patients with chronic inflammation (Gabay 2006) such as rheumatoid arthritis, Chrohn's disease, pulmonary tuberculosis and leprosy. Over production of this cytokine can also lead to plasmocytosis which can lead to tumours/cancers (Hirano 1991).

1.5.4 Interleukin-8

This cytokine is part of the chemokine family, it is also known as *CXCL8*. The gene is found in a chemokine gene cluster on chromosome 4. It is a pro-inflammatory cytokine that acts as a chemotactic factor that attracts neutrophils, basophils and T-cells to the site of inflammation. Macrophages, epithelial cells and platelets secrete the cytokine to initiate activation and chemotaxis (Luster 1998). Chemokines play a vital role in inflammation and phagocytosis, and primarily function in the innate immunity (Luster 1998). Anit-IL8 antibodies are used in treatment of inflammatory diseases (Yang *et al.* 1999).

1.5.5 Interleukin-10

IL-10 mediates both the adaptive and innate immune response. It is however primarily involved in the humoral immune response and is known for its anti-inflammatory properties. The cytokine induces differentiation of naïve T-cells to T_H2 cells, it promotes IL-4 production which promotes differentiation of additional T_H2 cells in a positive feedback loop (Mulligan *et al.* 2000). Ultimately, IL-10 leads to B-cell growth and differentiation (Go *et al.* 1990). As it functions to promote the T_H2 pathway, it simultaneously aims to inhibit the T_H1 response pathway. Overall, this cytokine plays a major suppressive role in the immune response (Del Prete *et al.* 1993; Sieling *et al.* 1993). It acts to inhibit cellular activity via preventing antigen presentation and cytokine production (Fiorentino *et al.* 1991). *IL-10* knock-out mice develop severe colitis due to overstimulation of the T_H1 immune response (Fiorentino *et al.* 1991). Due to its immunosuppressive and anti-inflammatory function, the cytokine is used in many treatments for chronic inflammation.

1.5.6 Colony-stimulating factor 2

CSF2 is found on chromosome 17 and is in an important pro-inflammatory cytokine that is primarily involved in the innate immune response (Gasson 1991). It is a growth factor for hematopoietic cells and stimulates the growth and maturation for various immune cells within the innate immune system such as macrophages dendritic cells (Metcalf 1986; Gasson 1991). This cytokine has been closely associated with many inflammatory diseases such as arthritis, inflammatory lung disease and inflammatory renal disease (Hamilton 2008).

1.5.7 Interferon-gamma

The last cytokine that is of particular importance for this study is IFN-γ. This cytokine is secreted by cells in the innate and adaptive immunity. It promotes antigen processing and presentation and plays an important role in both antiviral (cellular; Ijzermans and Marquet 1989; Samuel 2001) and antibacterial (humoral; Snapper *et al.* 1988) immune responses. IFN-γ also plays an important role in the innate immunity through its promotion of inflammation as well as increasing antigen MHC class I and II presentation on a variety of cells, especially macrophages (Bishop *et al.* 1986; Suto and Srivastava 1995). The cytokine's involvement in the innate and both adaptive immune responses makes it a highly pleiotropic cytokine.

1.5.8 Summary of selected cytokines

Cytokines are the proteins that monitor the intensity and duration of the complex immune response. It has been established that interleukin -6, -8 and CSF2 function mainly in the innate immunity and interleukin -4, -2, -10 and IFN-γ function primarily in the adaptive immune system. Interleukin 4 and 10 are primarily anti-inflammatory and interleukin 6 and TNF-α primarily pro-inflammatory. However, it is evident that cytokines perform a variety of functions that overlap with one another and most of the cytokines function as both pro- and anti-inflammatory, as well as play a role in the innate and adaptive, and cellular and humoral immune responses (Vilček 2003).

Polymorphisms at cytokine-related genes are an important field of research; they have been shown to influence cytokine functionality (Smith and Humphries 2009) and immune response (Poland *et al.* 2007). Some examples of this can be observed in Table 2.1

1.6. Conclusion and Aim

Most studies investigating the association between facial appearance and health as well as the genetics in facial appearance are performed in Western, educated, industrialised, rich and democratic (WEIRD) populations, this is unrepresentative of the human population in general (Henrich *et al.* 2010). By expanding research into other populations such as the African population, we obtain a more global understanding of facial appearance and its relationship with health. The relationship between facial appearance and immunity (as measured by response to hepatitis B-vaccination, cytokine response and HLA heterozygosity) is more strongly supported in men than in women (Roberts *et al.* 2005; Coetzee *et al.* 2007; Lie *et al.* 2008; Lie *et al.* 2010; Rantala *et al.* 2013a; Rantala *et al.* 2013b; Phalane *et al.* 2017). Facial appearance is highly genetically driven (Djordjevic *et al.* 2016) and is influenced by health (Henderson *et al.* 2016), but to my knowledge only the HLA genes have been studied to assess the genetic link between facial appearance and health in non-clinical populations. The HLAs only represent a small fraction of the immune system. Cytokines and their polymorphisms are a good representation for the functioning of the entire immune system, and their activity largely influences an individual's health (Hollegaard and Bidwell 2006; Smith and Humphries 2009). **Therefore, the aim of the proposed study is to assess the link between genetic variation at seven selected cytokine loci and facial appearance in a non-clinical cohort of African males.**

Chapter 2

2.1. Introduction

"A man's face is his autobiography"-Oscar Wilde

Facial appearance forms an integral part in everyday interactions as it reveals a wealth of information about the individual, starting from the very foundation that makes us who we are; our genetics. Facial appearance is shaped from many environmental factors however, it does have a very strong genetic component (Djordjevic *et al.* 2016) with narrow-sense heritability estimates as high as r=0.8 (Johannsdottir *et al.* 2005; Weinberg *et al.* 2013; Cole *et al.* 2017). There has been a relative lack of genetic studies assessing facial variation in the non-clinical populations. However, this is now changing with the improvement of facial imaging techniques as well as the advancement of gene sequencing and the development of GWAS studies.

GWAS and candidate gene studies have both been used to assess the genetic make-up of the face in non-clinical populations and there are advantages and pitfalls to both approaches. GWAS studies are advantageous as it offers an unbiased, hypothesis-free approach to finding many genetic variants across the entire genome (Pearson and Manolio 2008) that could regulate facial appearance. However, because GWAS studies are more of an exploratory approach, there is a very high degree of multiple testing and therefore a high false discovery rate; this could lead to less 'powerful' results (Sham and Purcell 2014). Significant findings in well-designed candidate gene studies can be more conclusive and powerful than in GWAS studies (Jorgensen *et al.* 2009). Careful consideration must however be taken when choosing candidate genes, and the sample cohort for the study must be large enough and as uniform as possible in terms of age, ethnicity and gender (Jorgensen *et al.* 2009; Wilkening *et al.* 2009). Both GWAS (Liu *et al.* 2012; Paternoster *et al.* 2012; Adhikari *et al.* 2016; Cole *et al.* 2016; Qiao *et al.* 2016; Shaffer *et al.* 2016) and candidate gene studies (Boehringer *et al.* 2011; Peng *et al.* 2013; Claes *et al.* 2014b; Peng *et al.* 2016) have been utilised to find the genes that significantly influence facial appearance in the non-clinical populations.

Variation in facial appearance is obtained and measured in a variety of ways. Facial images are captured as 3D or 2D images. 3D images capture the most variation in the face however obtaining 3D images of patients or participants can be very costly and

challenging and analysing 3D images is computationally intensive (Peng *et al.* 2013; Claes *et al.* 2014b). Therefore 2D images are still widely used in facial appearance studies. Facial images are often analysed through plotting limited landmarks manually on the face, gathering their positions, and measuring the distances between these landmarks (Liu *et al.* 2012; Adhikari *et al.* 2016; Cole *et al.* 2016; Peng *et al.* 2016; Shaffer *et al.* 2016). A substantial amount of variation in the face is however lost through this method. A principal component analysis (PCA) of standardised x and y co-ordinates of landmark points plotted on the face can also be conducted (Liu *et al.* 2012; Paternoster *et al*. 2012); this allows for the inclusion of many more points to be plotted on the face and therefore greater variance in the face is explained (Gottumukkal and Asari 2004). A PCA is a dimensional reduction statistical method that groups data points that are closely correlated into principal components (PCs; Abdi and Williams 2010).

Facial imaging has been a very important tool in the forensic sciences (Peng *et al.* 2013; Claes *et al.* 2014b), evolutionary studies (Little *et al.* 2011) and even in the clinical field where facial imaging can aid in the diagnosis of various health issues and genetic conditions (Hammond *et al.* 2004; Mutsvangwa *et al.* 2010; Lucassen *et al.* 2011; Ferry *et al.* 2014). Currently, there is also an ongoing development in advanced mirrors that can detect subtle changes in skin colouration and adiposity levels in the face; through this, certain cardiovascular and metabolic syndromes as well as immune functioning can be detected from the faces presented in the mirror (Poh *et al.* 2011; Colantonio *et al.* 2015; Re and Rule 2016).

Relevant health information can be gathered from the face through imaging analysis using sophisticated software. However, a great deal of accurate health information about an individual can be gathered from the everyday observations and judgements we make based on facial appearance. Humans can perceive a substantial amount of information about an individual from only their facial appearance; from obvious traits such as gender, age and ethnicity as well as more abstract traits such as IQ (Zebrowitz *et al.* 2002; Talamas *et al.* 2016), personality (Hassin and Trope 2000; Little *et al.* 2006), attractiveness (Langlois *et al.* 2000) and health status (Zebrowitz and Rhodes 2004) of an individual. What is extraordinary is the agreement across individuals and the accuracy above just chance of the judgements made on an individual when the only information provided is a neutral-looking face (Kalick *et al.* 1998; Zebrowitz and Rhodes 2004; Penton-

Voak *et al.* 2006; Kramer and Ward 2010; Kleisner *et al.* 2014). In this study, we are particularly interested in how the face reveals the health of an individual.

There are common facial cues, already mentioned in chapter one, that have shown to be associated with health appearance and the actual health of a person. Most commonly; faces that are closer in resemblance to the population average (Thornhill and Gangestad 1999; Rhodes *et al.* 2001), are more symmetrical (Thornhill and Gangestad 2006; Gangestad *et al.* 2010), more masculine in men or feminine in women (Rhodes *et al.* 2003; Thornhill and Gangestad 2006; Smith *et al.* 2009; Rantala *et al.* 2013a), have lighter, yellower and redder skin tones (Stephen *et al.* 2009b; Stephen *et al.* 2011; Henderson *et al.* 2017; Phalane *et al.* 2017) and have adiposity (fat) levels that correspond to a normal BMI (Coetzee *et al.* 2009; Tinlin *et al.* 2013; Henderson *et al.* 2016; Foo *et al.* 2017a), represent better health. These facial cues have been linked to various indirect and direct measures of immunity. Indirect measures include self-reported health measures; this involves medical records of participants as well as recollection of various health indicators, number of infections, antibiotic use etc. Facial symmetry, averageness, adiposity and masculinity/femininity have all been associated with immunity through self-reported measures of health (Rhodes *et al.* 2001; Thornhill and Gangestad 2006; Coetzee *et al.* 2009; Boothroyd *et al.* 2013). A few studies have investigated more direct measures of immunity, such as antibody response to vaccination and cytokine response, which is needless to say a more reliable indicator of immunity. Response to hepatitis B vaccinations have been positively associated with masculinity and negatively with adiposity (Rantala *et al.* 2013a). Cytokine response has been positively and significantly associated with a lighter and yellower skin tone as well as increased masculinity and adiposity in predominantly under-to-normal weight men. (Phalane *et al.* 2017). Skin colour changes have been reported after stimulation of lipopolysaccharides to mimic the effects of acute infections on skin colour (Henderson *et al.* 2017).

The face is highly heritable and has been shown to be a good representation of certain aspects of an individual's health, particularly immunity. However, not many studies have been conducted to assess the genetic link between facial appearance and health. The only studies to our knowledge, as previously mentioned in chapter one, have investigated the influence that HLA genes have on facial appearance (Roberts *et al.* 2005; Coetzee *et al*. 2007; Lie *et al.* 2008).

The relationship between facial appearance and measures of immunity has been investigated through HLA studies (Roberts *et al.* 2005; Lie *et al.* 2008), response to hepatitis B vaccinations (Rantala *et al.* 2013a), self-reported health measures (Rhodes *et al.* 2001; Thornhill and Gangestad 2006; Coetzee *et al.* 2009; Boothroyd *et al.* 2013), oxidative stress levels (Gangestad *et al.* 2010) and cytokine response (Phalane *et al.* 2017). However there are a number of gaps still present in the literature. The immune system is a complex system with various response pathways and functions, depending on the invading body. It is divided into the innate and adaptive immune response (Parkin and Cohen 2001). The innate immunity is the body's immediate response to a foreign antigens present in the body (Beutler 2004). The adaptive immunity is a learned immune response that is specific and produces a memory. The adaptive immune response is further divided into the T_H1 (cellular) and T_H2 (humoral) immune response pathways (Abbas *et al.* 2016).

Cytokines are good candidates to investigate the genetic link between facial appearance and health. Cytokines coordinate the entire immune response and are involved in both the innate and adaptive immunity (Vilček 2003). This study focuses on polymorphisms found in cytokines primarily involved in the T_H1 immune response (*IL-2* and *IFN-γ*), the T_H2 immune response (*IL-4, IL-6* and *IL-10*; Romagnani 1999) and across both the innate and adaptive immune responses (*IL-8* and *CSF2*; Shi *et al.* 2006; Mantovani *et al.* 2011). Polymorphisms within cytokine-related genes have been shown to influence cytokine functionality (Smith and Humphries 2009), immunity and health (Poland *et al.* 2007). A few examples for this can be observed in Table 2.1.

Therefore the aim of this study is to assess the association between cytokine-related SNPs and facial appearance. The study was conducted in African men as the African population (particularly a homogenous African population) has been relatively under-represented in the literature concerning facial genetic studies as well as studies concerning health and facial appearance. We chose to conduct the study in men because the relationship between facial appearance and health, particularly immune response and genetic measures of immunity, seems to be more prominent in men compared to women (Coetzee *et al.* 2007; Lie *et al.* 2008; Lie *et al.* 2010; Rantala *et al.* 2013a; Phalane *et al.* 2017). The study is divided into two experiments (Figure 2.1); in experiment one we assessed the association that cytokine SNPs have with health perception and the facial

cues that influence health perception (masculinity, adiposity, skin colour, averageness and symmetry). In experiment two, we assessed the association that cytokines have with the structural components of the face. The significant structural components were then characterised by assessing their relationships with the facial perceptions obtained in experiment one and observing their average faces of the low and high structural components.

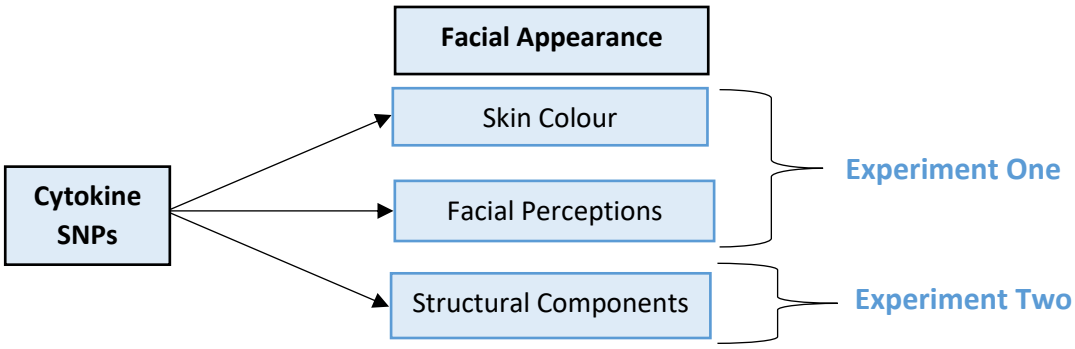


Figure 2.1. *An overview of how this study will assess the relationship between cytokine SNPs and facial appearance*

2.2 Methods and Materials

2.2.1. Collecting data

Cytokine SNP selection

An extensive literature and database search was conducted primarily on the National Centre for Biotechnology Information (NCBI) and Ensembl websites under the SNP databases as of Aug-Sept 2015, and associated literature. Seven cytokines were chosen, including interleukins, (*IL-2, IL-4, IL-6, IL-8*, and *IL-10*), interferon-gamma (*IFN-γ*) and colony-stimulating factor 2 (*CSF2*). These cytokines were chosen because they were well studied and collectively, they represent many aspects of the immunity including both anti-inflammatory and pro-inflammatory processes, the innate and adaptive, as well as the cellular and humoral immune responses (Hanada and Yoshimura 2002; Thomson and Lotze 2003). In order to be included in the candidate list, SNPs associated with these 7 cytokines had to meet the following criteria: (a) the SNPs had to have a minor allele frequency (MAF) of >25% in the combined African populations, obtained from the 1000 genomes project (ref: GRCh37.p13); (b) the SNPs should have been extensively studied and cited; and (c) the SNPS should have been previously associated with cytokine levels

or immune-related diseases and health. Nine cytokine-associated SNPs were selected and included in a custom TaqMan® OpenArray® Plate designed by Applied Biosystems® (Table 2.1).

Sample collection

Ethical approval was obtained in writing from the ethics committee at the University of Pretoria (EC141002-083). African men (N=95, mean age= 20.5 yrs., SD = 2.93) were recruited from the Mamelodi and Hatfield campuses at the University of Pretoria. Informed consent was gathered and questionnaires were completed by all participants to gather basic demographic information, such as age and ethnicity. Participants were made aware that they did not have to complete any questions they were not comfortable answering and they were allowed to leave the study at any time. Physical measurements were recorded for each participant. Height was recorded in centimetres and weight was measured on a Camry® body-fat scale. This scale also uses age, height, gender and the weight it measures, to calculate body fat percentage. Each participant's BMI was calculated using their weight and height (kg/m^2).

Skin colour measurements were taken using a Konica Minolta CM-2300d spectrophotometer. The spectrophotometer measures skin colour in the CIELab colour space: CIELab L* (luminance axis), CIELab a* (red-green axis) and CIELab b* (blue-yellow axis). Colour measurements were taken on the right cheek, left cheek and forehead. Each point was measured twice and the average calculated. We were conscious to choose areas that were free from moles or other skin blemishes while taking the skin colour measurements.

Facial photographs were captured using a Canon EOS 40D camera with 60mm f/2.8 macro lens in a photo booth with a white background. Lighting was provided by three daylight simulation fluorescent bulbs within the photo booth; two on each side and one above. Participants were seated about two meters from the camera and asked to maintain a neutral facial expression. They were first asked to look straight at the camera when capturing the frontal facial photograph and then turned and faced the right of the booth when taking the profile photo. Two sets of photos were taken for both the frontal and profile facial images for each participant. The best quality frontal and profile images for each participant were used for further analyses.

Participants were asked to return a few weeks later for blood collection which was performed by a trained phlebotomist. Blood was collected from only 71 participants in 4 ml EDTA tubes. The blood was aliquoted into smaller volumes and stored at -20°C.

For the purpose of this MSc only participants who's DNA could be obtained via blood samples were included in the study. The final sample size used in this study was therefore 71 African men (mean age= 20.37 yrs., SD = 2.78).

2.2.2. Facial imaging

Standardising facial Images

JPsychomorph from Java was used to delineate a frontal and profile image for all participants (Tiddeman and Perrett 2001). This involves plotting 179 and 53 points on the frontal and profile faces respectively (Figure 2.2 A & B). The JPsychomorph software was used to also standardise the images by cropping them, to remove most of the background and clothing, as well as aligning them using a Procrustes alignment.

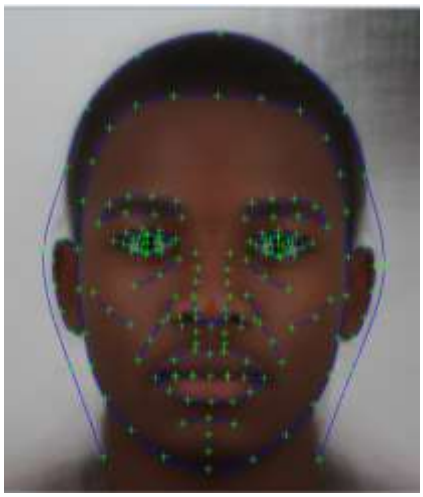
A.  **B.** 

Figure 2.2. *One hundred and seventy nine and 53 points plotted on the average (A) frontal and (B) profile faces respectively using JPsychomorph*

Determining perceptual judgements of the faces

The standardised frontal facial images were used to create a computer based test in which the faces appeared on the screen, one at a time, in a random order and each face could be rated on a 7-point Likert scale. Five separate tests were created to gather different

ratings for all the faces. These ratings involved judgements on the male faces based on their adiposity (1=very underweight to 7=very overweight), health appearance (1=very unhealthy to 7= very healthy), their distinctiveness (1=not distinctive to 7=very distinctive), symmetry (1=very unsymmetrical to 7= very symmetrical) and finally masculinity (1=not very masculine to 7=very masculine). African females (N=20, mean age= 20.5 yrs.) were recruited to rate the male faces for each test. The distinctiveness score was reverse coded to produce an averageness score. An average adiposity, perceived health, averageness, masculine and symmetrical score was then calculated for each male participant.

Determining structural components of the face

We performed a small pilot study in order to identify the most reliable delineation points for structural analysis. To identify these points, three faces were randomly chosen and each face was delineated three separate times. For each face, the difference in positioning for each point was calculated using the extracted x- and y- co-ordinates. Facial landmark points (a) with the lowest relative deviation between the different delineation attempts and (b) were placed on important structural facial features, were included in subsequent analysis. This process was conducted for both the frontal and profile facial images. The landmark points were reduced to 48 frontal and 23 profile points. JPsychomorph was used to reduce the number of points on the standardised frontal and profile templates (Figure 2.3 A & B). Both X and Y co-ordinates were obtained from the reduced set of 48 frontal and 23 profile points. Many of the remaining frontal and profile points were highly correlated so we performed a PCA in "*IBM SPSS Statistics version 20"* to identify fewer, uncorrelated components.

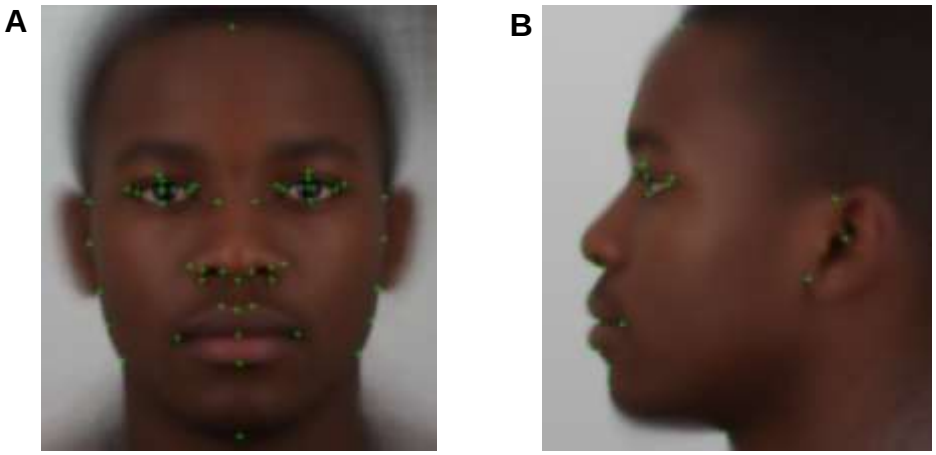


Figure 2.3. *The reduced set of points used for structural analysis of the face. Forty-eight and 23 points were plotted on the average (A) frontal and (B) profile faces respectively*

2.2.3. Genotyping

DNA extraction

Blood samples were defrosted at room temperature. Genomic DNA (gDNA) was extracted from 200 ul of the whole blood sample using the Quick-DNA™ Universal kit (from *Zymo Research*), following manufacturer's "biological fluids and cells" protocol. The purified DNA's quality and concentration was measured using a Nanodrop 2 000 Spectrophotometer; the 260/230 ratio, 260/280 ratio and DNA concentrations in ng/ul were recorded. The gDNA was also analysed on a 1% agarose gel to ensure the DNA was intact. The gDNA was diluted into 50 ng/ul solutions and stored at -20 °C.

Genotyping

The samples (N=71) were genotyped using the *Applied Biosystems® by Life Technologies™ QuantStudio 12K Flex Real-Time PCR System*. All gDNA (50 ng/ul) samples were of sufficient quality according to the "Applied Biosystems™ Quantstudio™12K Flex Real-Time PCR System user guide" (260/230 and 260/280 ratios between 1.6 and 2). The protocol was followed according to the manufacturer's instructions for genotyping ("Applied Biosystems™ Quantstudio™12K Flex Real-Time PCR System user guide"). The Genotyping-128 format was used which allowed for all samples to be genotyped in duplicate.

The gDNA samples and the custom design *TaqMan® OpenArray®* Plate were defrosted at room temperature. DNA (2.5 ul of 50 ng/ul samples) and *TaqMan® OpenArray®* Genotyping Master Mix (2.5 ul) was prepared into each well of the 384-well sample plate. Samples were transferred from the 384-well plate to the *TaqMan® OpenArray®* Plate using the automatic *Accufill™* system from *Applied Biosystems™*. Sample positions in the wells were tracked using the *OpenArray® Sample Tracker* software. The *QuantStudio™ 12k Flex* software was prepared, the *TaqMan® OpenArray®* Plates containing samples were loaded into the *QuantStudio™ 12K Flex System* and the experiment ran over night.

2.2.4. Statistical analysis

Genetic analysis

The nine candidate SNPs that were genotyped in 71 samples were individually analysed on the Taqman Genotyper Software from Applied Biosystems to assess their quality. All

nine SNPs had an assay call rate >90% and a minor allele frequency MAF >17% (Table 2.1). The genotypes for each SNP were then coded assuming an additive genetic model.

Facial analysis

Perception data was tested for inter-rater reliability and normality. Inter-rater reliability was tested through calculation of Cronbach alpha values. Skewness and kurtosis values for each of the 5 perceptual judgements (health, adiposity, averageness, symmetry and masculinity) were then calculated to ensure a normal distribution of the variables. Relationships between the perceptions were assessed using a Pearson's correlation (Table 2.3).

To reduce the number of variables and prevent redundancy in the subsequent associations, separate PCAs were performed on the perceptual facial cues (Table 2.4) and colour measurements (Table 2.5) that influence a healthy appearance. Both perceptual and colour PCs were extracted from the dimension reduction analysis. Structural PCs were also gathered from a subset of reliable points on the frontal and profile faces that were identified in section 2.3 (Table 2.6).

Final analysis for experiment 1 and experiment 2

Figure 2.1 summarises the analyses that were used to assess the relationship between cytokine related SNPs and facial appearance variables. The analyses were divided into two experiments. In both experiments one and two, separate linear regressions were performed and the nine cytokine SNPs were used as the independent variables. The dependant variables in experiment one were overall health appearance and the facial cues that influence health appearance (principal components of skin colour and perceived masculinity, adiposity, symmetry and averageness). The dependant variables in experiment two were seven structural PCs. The significant structural PCs from experiment two were characterised to assess how they explain facial shape. The majority of analyses were performed in *IBM SPSS Statistics version 20*.

2.3 Results

2.3.1. Genotypic Data

Literature search

Table 2.1 shows the results after an extensive search primarily made through the NCBI and Ensembl databases for nine candidate SNPs. All SNPs chosen had a MAF >25% in the African population and had preferably shown significant relationships with cytokine levels in previous studies or had at least shown significant associations with immune-related diseases or cancers. All of the selected SNPs were localised within the transcribed regions of their associated cytokine genes, except for a SNP near *CSF2* which is found in the enhancer site.

Table 2.1. *Candidate SNPs that were selected for the study and the rationale for choosing these specific variants*

Gene	dbSNP	SNP location	MAF *	Examples of Phenotypic Associations
IL-2	**rs11932411 (T/C)**	3' UTR	0.38 (C)	Showed an allele dose related increase/decrease of antibody levels in measles vaccination in African Americans (p=0.028; Haralambieva *et al.* 2011)
IL-4	**rs2070874 (T/C)**	Promoter (-34)	0.48 (T)	The IL-4 promoter SNP has shown to influence IL-4 expression and protein production when studied within a haplotype group (Anovazzi *et al.* 2017) or when studied as a single polymorphism (Nakashima *et al.* 2002; Gonzales *et al.* 2010). The interleukin 4 promoter SNP has also shown associations with patients that have asthma and atopy (Rosenwasser and Borish 1997; Smolnikova *et al.* 2013).
IL-6	**rs10499563 (T/C)**	Promoter (-6331)	0.23 (C)	The SNP causes a change for DNA binding proteins and as a result, the T allele is associated with an increase in IL-6 gene expression and IL-6 plasma levels (Smith *et al.* 2008)
	rs2069845 (G/A)	Intron 4 (+869)	0.32 (G)	The SNP alters restriction enzyme recognition sites and has been shown to influence the relationship between diet and obesity in South African woman (Joffe *et al.* 2014). The G-allele has been associated with a risk for developing obesity in children and adolescents (Todendi *et al.* 2015). And the allele also increases the odds of developing a symptom cluster (of

				pain, fatigue, sleep disturbance and depression) which develops due to a release of pro-inflammatory cytokines in cancer patients (Doong *et al.* 2015).
IL-8	**rs2227307 (T/G)**	Intron 1 (+396)	0.48 (T)	The T allele is a significant risk factor for chronic periodontitis (Zhang *et al.* 2014). The SNP is in strong LD and forms a haplotype group with rs4073 and rs227306 (Slade *et al.* 2011; Zhang *et al.* 2014), which has been associated with IL8 levels (Hull *et al.* 2001; Hacking *et al.* 2004). The SNP is also significantly associated with idiopathic pulmonary fibrosis (Ahn *et al.* 2011) and survival rates in cancers (Aschebrook-Kilfoy *et al.* 2012; Slattery *et al.* 2014).
IL-10	**rs1800896 (T/C)**	Promoter (-1082)	0.31 (C)	This SNP forms part of a haplotype group with two other SNPs (GCC) that has been associated with increased IL-10 production in vivo (Turner *et al.* 1997), stronger transcriptional activity (Crawley *et al.* 1999) and higher mRNA expression (Suarez *et al.* 2003). The C allele is associated with an increase of IL-10 mRNA expression as well as IL-10 ex-vivo and serum protein levels (Suarez *et al.* 2003).
IFN-γ	**rs2069705 (G/A)**	Promoter (-1616)	0.47 (G)	It has been associated with breast cancer (Erdei *et al.* 2010) as well as susceptibility to systemic lupus erythematosis (Kim *et al.* 2010; Leng *et al.* 2016) and complicated skin and skin structure infections (Stappers *et al.* 2014). It has been suggested to be associated with differential levels of IFN-γ although its effect on actual expression has not been studied directly (Kim *et al.* 2010).
CSF2	**rs25882 (T/C)**	Ile117Thr	0.32 (C)	This SNP (T allele) has been associated with asthma (Rohrbach *et al.* 1999) and higher allelic expression of CSF2 (Burkhardt *et al.* 2012).
	rs721121 (T/G)	Enhancer site	0.33 (G)	The SNP is found in the enhancer site where an intergenic transcription factor binds to regulate CSF2 and IL-3 expression in T-cells (Cockerill *et al.* 1993)

**the minor allele frequencies (MAF) were those found in the African population from the 1000 genomes project (ref: GRCh37.p13).*

Quality control of SNPs

Table 2.2 reveals that all nine SNP had assay call rates >90% and a MAF >17%. Not all samples were successfully genotyped for all SNP assays therefore each SNP sample size differs slightly, according to the assay call rate.

Table 2.2. *Success rate of allele assays and minor allele frequencies (MAF) after genotyping.*

Gene	SNP	Assay call rate (%)	Sample size	MAF (%)
IL2	rs11932411	97.2	69	35.5
IL4	rs2070874	100	71	42.3
IL6	rs10499563	95.8	68	19.1
	rs2069845	100	71	26.1
IL8	rs2227307	98.6	70	42.9
IL10	rs1800896	90.1	64	28.1
IFN-γ	rs2069705	97.2	69	27.5
CSF2	rs25882	98.6	70	17.1
	rs721121	100	71	41.5

2.3.2. Facial results

Facial perceptions

A Cronbach alpha value of >0.7 is generally considered an acceptable reliability co-efficient (Nunnally 1967). The 20 observers were consistent and reliable in their ratings of the 71 participants as all perceptual ratings had a Cronbach alpha value >0.7. The facial adiposity ratings were also significantly associated with BMI ($r=0.647$, $p<0.0005$), providing further evidence of the accuracy of the adiposity ratings (Figure 2.4). According to the World Health Organisation (WHO) standards, 17% of the participants were underweight (BMI<18.50 kg/m^2), 66% of the participants were normal weight (BMI between18.50-24.99 kg/m^2) and 17% of the participants were overweight (BMI>25 kg/m^2; Organization 2000). All perceptual ratings were normally distributed (two-tailed critical z-score = ± 3.29, p = 0.001). Pearson's correlations were performed to identify significant relationships between health perception and the perceptual cues that may influence a healthy appearance (Table 2.3).

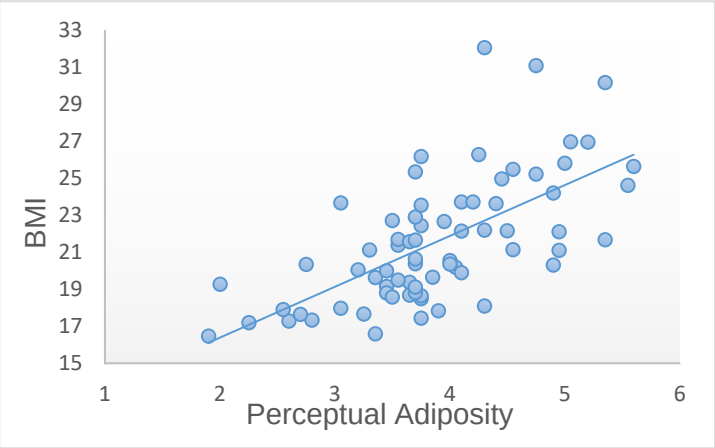


Figure 2.4. *A graph representing the close linear relationship between body mass index and perceptual adiposity*

Table 2.3. *Pearson's correlations between all facial perceptions*

	Averageness	Adiposity	Masculinity	Symmetry
Health	0.440**	0.202	0.558**	0.602**
Averageness	1	-.0041	0.085	0.264*
Adiposity	-	1	0.451**	0.039
Masculinity	-	-	1	0.536**

*p<0.05, **p<0.01, ***p<0.001

As expected, health appearance was strongly, positively correlated with facial averageness, masculinity and symmetry (Table 2.3). The relationship between health and adiposity did not show a significant linear correlation (Table 2.3). However upon further inspection, a significant curvilinear relationship was found between health and adiposity ($F_{2, 69}$= 7.178, p=0.001, R^2=0.174). This is observed in Figure 2.5 which reveals that adiposity has an optimum level of health appearance and adiposity ratings that decrease and increase from this optimum are found to appear less healthy. The optimum adiposity score that appears the healthiest on a 7-point Likert scale is ~ 3.5 (Figure 2.5), which corresponds to a BMI of ~ 20 kg/m^2 (Figure 2.4).

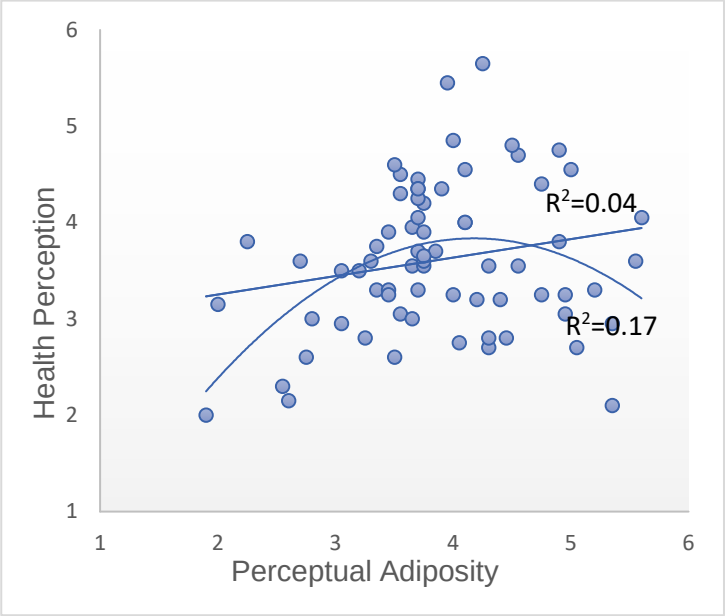


Figure 2.5. *A graph comparing the linear and curvilinear relationship between adiposity ratings and their influence on health ratings*

Many of the facial cues that influence a healthy appearance (adiposity, symmetry, masculinity and averageness) are significantly and positively correlated to one another (Table 2.3). A non-linear relationship was observed between masculinity and facial adiposity. A quadratic relationship between facial adiposity as the dependant variable and masculinity as the independent variable explained the most variance ($F_{2, 68}$=19.585, p<0.001, R^2=0.365), followed by a quadratic relationship between masculinity as the dependant variable and facial adiposity as the independent variable ($F_{2, 69}$= 12.884, p<0.0005, R^2= 0.275), and a linear relationship between facial adiposity and masculinity ($F_{1, 69}$=17.615, p<0.0005, R^2=0.203; Figure 2.6). The quadratic relationship between facial adiposity and masculinity was even more significant after the removal of one influential outlier ($F_{2, 67}$=23.315, p<0.0005, R^2=0.410; Figure 2.6). To include this quadratic relationship linearly, both masculinity^2 and adiposity^2 variables were added for subsequent analyses. None of the other facial perceptions showed a non-linear relationship with health or with each other.

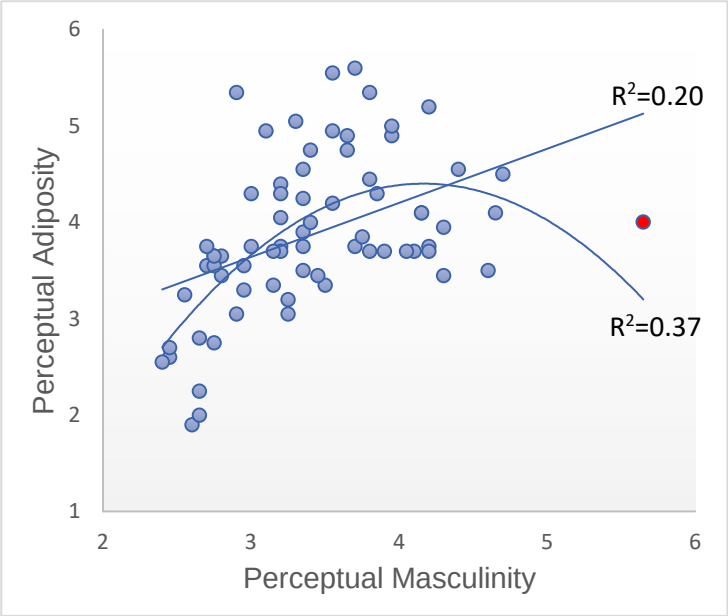


Figure 2.6. *A graph comparing the linear and curvilinear relationship between masculinity ratings and their influence on adiposity ratings. This relationship remained significant after the removal of the possible outlier (highlighted in red)*

A PCA was performed on the collective facial cues (adiposity, masculinity, symmetry, averageness, masculinity^2 and adiposity^2) that influence health perception. The averageness variable was not highly extracted (only 24.2% of the variable was explained in the subsequent PCs from the PCA). Averageness was therefore removed from the PCA and analysed as a separate variable.

The new PCA (excluding averageness) resulted in two PCs that collectively explain 90% of the total variance. Masculinity and adiposity loaded highly and positively on PC 1, which explains 59% of the total variance. Symmetry (positively) and adiposity (negatively) loaded highly on PC 2, which explains 31% of the total variance. Therefore PC 1 was referred to as the +masculinity/+adiposity PC and PC 2 was identified as the +symmetry/-adiposity PC (Table 2.4).

Table 2.4. *The component matrix after a principal component analysis, excluding averageness*

	Component Matrix	
	PC 1	**PC 2**
Adiposity	**0.763**	**-0.628**
Masculinity	**0.907**	0.330
Symmetry	0.502	**0.668**
Adiposity^2	**0.731**	**-0.666**
Masculinity^2	**0.881**	0.375

Facial colour

The CIELab a* (green-red axis), CIELab b* (blue-yellow axis) and CIELab L* (luminance axis) skin colour readings for the right cheek, left cheek and forehead were collected twice using the skin colour spectrophotometer in a subset of 44 men. The two measurements for each point were averaged. Higher values of these measurements represent lighter (CIELab L*), redder (CIELab a*) and yellower (CIELab b*) readings. As in previous research on African populations, these measurements were all highly positively correlated with one another ($p<0.0005$; Coetzee *et al.* 2012; Phalane *et al.* 2017). To avoid using an excessive number of variables that would lead to redundant results, a PCA was conducted to reduce the number of colour variables. The PCA produced one colour PC that explains 83% of the total colour variance in the face (Table 2.5). Higher colour PC values indicate faces that are lighter, yellower and redder.

Table 2.5. *Component matrix after a principal component analysis using all CIELab colour measurements for the forehead, left cheek and right cheek*

	Component Matrix
	PC1
Forehead (L)	0.845
Forehead (a*)	0.887
Forehead (b*)	0.939
Left cheek (L)	0.933
Left cheek (a*)	0.854
Left cheek (b*)	0.960
Right cheek (L)	0.926
Right cheek (a*)	0.906
Right cheek (b*)	0.948

Facial structural components

The PCA conducted on the X and Y coordinates of the 48 and 23 points plotted on the frontal and profile faces respectively yielded a total of 13 PCs. Due to the small sample size of the study we decided to select a limited set of components from the PCA that explain at least 86% of the total variance in the face.

Table 2.6*. Structural principal components that were obtained after a principal component analysis was performed on the X and Y co-ordinates of the facial landmarks*

Component	**Eigen Values**	**% of Variance explained**	**Cumulative % of variance explained**
1	44.004	31.887	31.887
2	30.712	22.255	54.142
3	16.391	11.878	66.020
4	9.686	7.019	73.039
5	7.603	5.509	78.548
6	6.984	5.061	83.609
7	4.092	2.965	86.574

2.3.3. Experiment one analysis

The relationship between facial cues and healthy appearance

Healthy appearance was significantly and positively correlated with averageness (r=0.440, p<0.0005), PC 1 (+masculinity/+adiposity; r=0.525, p<0.0005), PC 2 (+symmetry/-adiposity; r=0.371, p=0.001) and the colour PC (r=0.464, p=0.002).

Adiposity loads positively on PC 1 and negatively on PC 2 (Table 2.4) and both PC1 and PC 2 are positively correlated to health. This is because of the curvilinear relationship between adiposity and health (Figure 2.5). As adiposity increases, so does a healthy appearance (possibly observed in PC 1) until an optimal weight is reached. In this experiment the relative BMI that appears healthiest is ~ 20 kg/m^2 (Figure 2.4 and 2.5), which lies perfectly in the healthy weight range as defined by WHO (18.5-25.0 kg/m^2; Organization 2000). As adiposity continues to increase into the overweight or obese range; healthy appearance decreases (possibly observed in PC 2). We wanted to assess if PC 1 truly represents the lower weight individuals and PC 2 represents the higher weight individuals. The dataset was split by the median for facial adiposity to form low and high "weight" groups. We assessed both groups' relationships with PC 1 and PC 2. As predicted, PC 1 showed a stronger, positive correlation with the low-weight group (r^2=0.77; p<0.0005) compared to the high weight group (r^2=0.37; p=0.029). And PC 2 showed a stronger negative correlation with the higher weight group (r^2=-0.68; p<0.0005) compared to the lower weight group (r^2=-0.16; p=0.35).

Health is significantly and positively correlated with PC colour (r=0.464, p=0.002). This means that individuals that have a lighter, redder and yellower skin tone appear to be healthier. As observed earlier in Table 2.3, health appearance is also significantly positively correlated with averageness, masculinity (found predominantly in PC 1) and symmetry (found predominantly in PC 2). To visualise the facial variables that will be used for further analyses in experiment one, composite images consisting of the average image of 10 faces were created for the highest and lowest values of each variable (Figure 2.7 A-E).

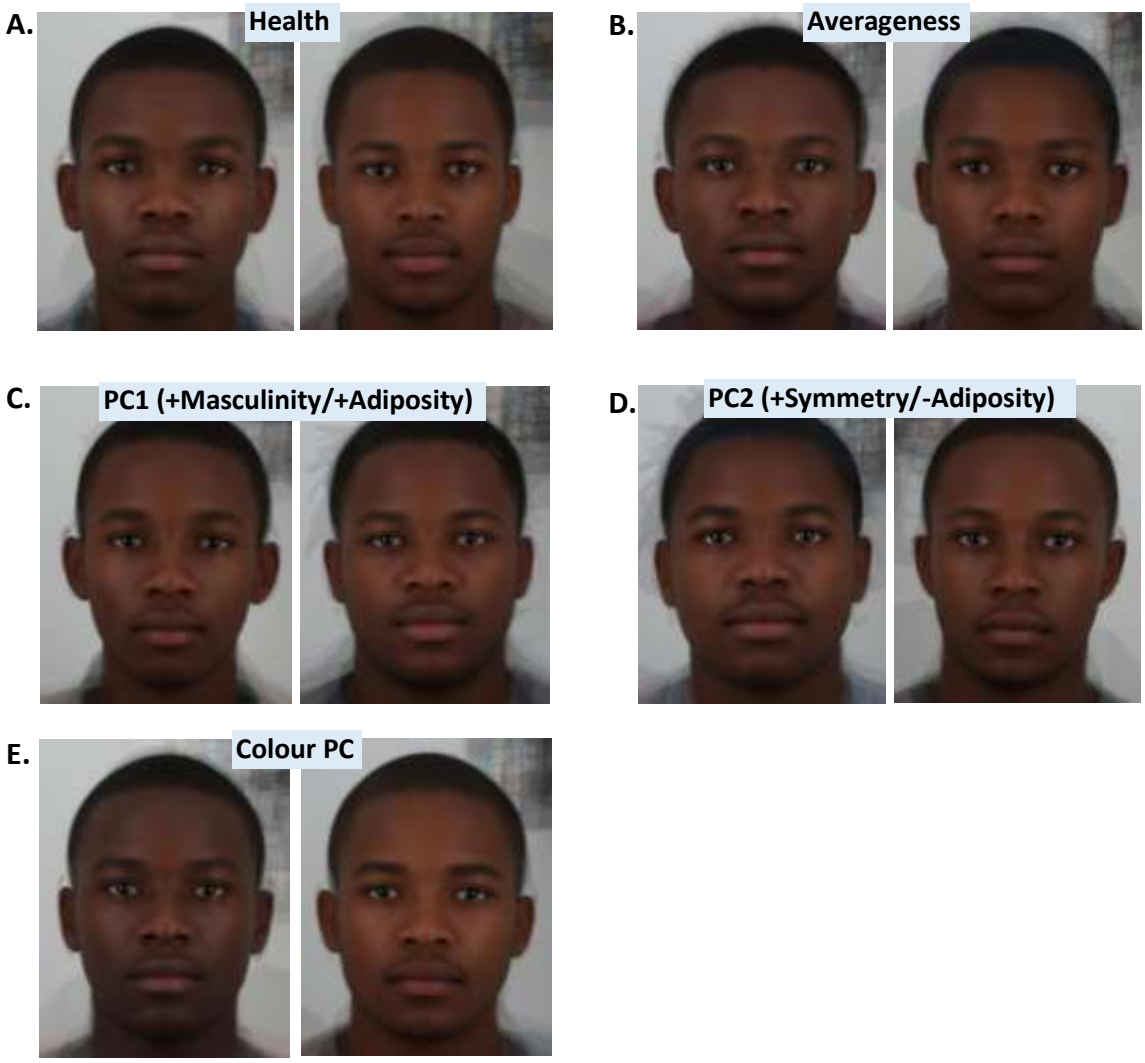


Figure 2.7. *Average faces of 10 participants with the lowest (images on the left) and 10 participants with the highest (images on the right) values for the respective variables [(A) health, (B) averageness, (C) +masculinity/+adiposity component, (D) +symmetry/-adiposity component and (E) colour component*

Linear regressions

Separate linear regressions were performed to assess the relationships between the genetic variation in SNPs (as the independent variable) and facial perceptions and colour (as the dependant variables). The results are shown in Table 2.7 and significant results are summarised in Figure 2.8.

From Figure 2.8 and Table 2.7, it can be observed that only rs1800896 (C/T SNP) in the *IL-10* promoter shows a direct significant relationship with health appearance; a healthier appearance is positively associated with the T allele frequency. The G allele in the

rs2227307 (G/T) SNP found in intron 1 of *IL-8* is significantly associated with higher +masculinity/+adiposity component values. The +masculinity/+adiposity component is highly positively associated with a healthy appearance. The SNP rs2070874, in the *IL-4* promoter is a C/T variant, the C allele is associated with higher colour PC values (lighter, yellower and redder skin tone). The colour PC is positively associated with apparent health. Finally, SNPs in the *IL-6* (rs2069845) and in *IFN-γ* (rs2069705) promoters are significantly associated with facial averageness. Rs2069705 in *IFN-γ* and rs2069845 in *IL-6* are A/G SNPs and individuals with the AA genotypes have significantly higher facial averageness ratings. Averageness is also highly positively associated with health perception. No SNPs were significantly associated with the +symmetry/-adiposity component. But the +symmetry/-adiposity component was significantly and positively associated with health.

Table 2.7. *Observed F statistics (the top number) and R^2 values (the bottom number) after separate linear regressions between SNP and facial appearance variables*

dbSNP	Health	Averageness	PC1	PC2	PC colour
IL-2 **rs2069771**	$F_{1,62}$ =0.182 R^2= 0.003	$F_{1,62}$ =0.311 R^2= 0.005	$F_{1,62}$ =0.906 R^2= 0.030	$F_{1,62}$ =0.993 R^2= 0.016	$F_{1,37}$=0.131 R^2= 0.004
IL-4 **rs2070874**	$F_{1,69}$ =0.804 R^2= 0.012	$F_{1,69}$ =0.104 R^2= 0.002	$F_{1,69}$ =0.086 R^2= 0.001	$F_{1,69}$ =0.104 R^2= 0.002	**$F_{1,42}$ =7.192**** **R^2= 0.146**
IL-6 **rs10499563**	$F_{1,66}$ =0.844 R^2= 0.013	$F_{1,66}$ =0.130 R^2= 0.002	$F_{1,66}$ =1.173 R^2= 0.017	$F_{1,66}$ =2.211 R^2= 0.032	$F_{1,40}$ =0.476 R^2= 0.012
IL-6 **rs2069845**	$F_{1,69}$ =0.491 R^2= 0.007	**$F_{1,69}$ =5.721*** **R^2= 0.077**	$F_{1,69}$ =0.341 R^2= 0.005	$F_{1,69}$ =0.030 R^2= 0.000	$F_{1,42}$ =0.031 R^2= 0.001
IL-8 **rs2227307**	$F_{1,68}$ =3.062 R^2= 0.043	$F_{1,68}$ =0.180 R^2= 0.003	**$F_{1,68}$ =10.685**** **R^2= 0.136**	$F_{1,68}$ =0.710 R^2= 0.010	$F_{1,42}$ =0.037 R^2= 0.001
IL-10 **rs1800896**	**$F_{1,62}$ =4.609*** **R^2= 0.069**	$F_{1,62}$ =0.011 R^2= 0.000	$F_{1,62}$ =2.442 R^2= 0.038	$F_{1,62}$ =1.009 R^2= 0.016	$F_{1,37}$ =0.727 R^2= 0.019
IFN-γ **rs2069705**	$F_{1,67}$ =0.365 R^2= 0.005	**$F_{1,67}$ =4.156*** **R^2=0.058**	$F_{1,67}$ =0.209 R^2= 0.003	$F_{1,67}$ =1.211 R^2= 0.018	$F_{1,41}$ =0.222 R^2= 0.005
CSF2 **rs25882**	$F_{1,68}$ =0.272 R^2= 0.004	$F_{1,68}$ =0.000 R^2= 0.000	$F_{1,68}$ =0.153 R^2= 0.002	$F_{1,68}$ =3.264 R^2= 0.046	$F_{1,41}$ =0.273 R^2=0.007
CSF2 **rs721121**	$F_{1,69}$ =0.202 R^2= 0.003	$F_{1,69}$ =0.432 R^2= 0.006	$F_{1,69}$ =0.000 R^2= 0.000	$F_{1,69}$ =0.919 R^2= 0.013	$F_{1,42}$ =0.160 R^2=0.004

*p<0.05, **p<0.01, ***p<0.001

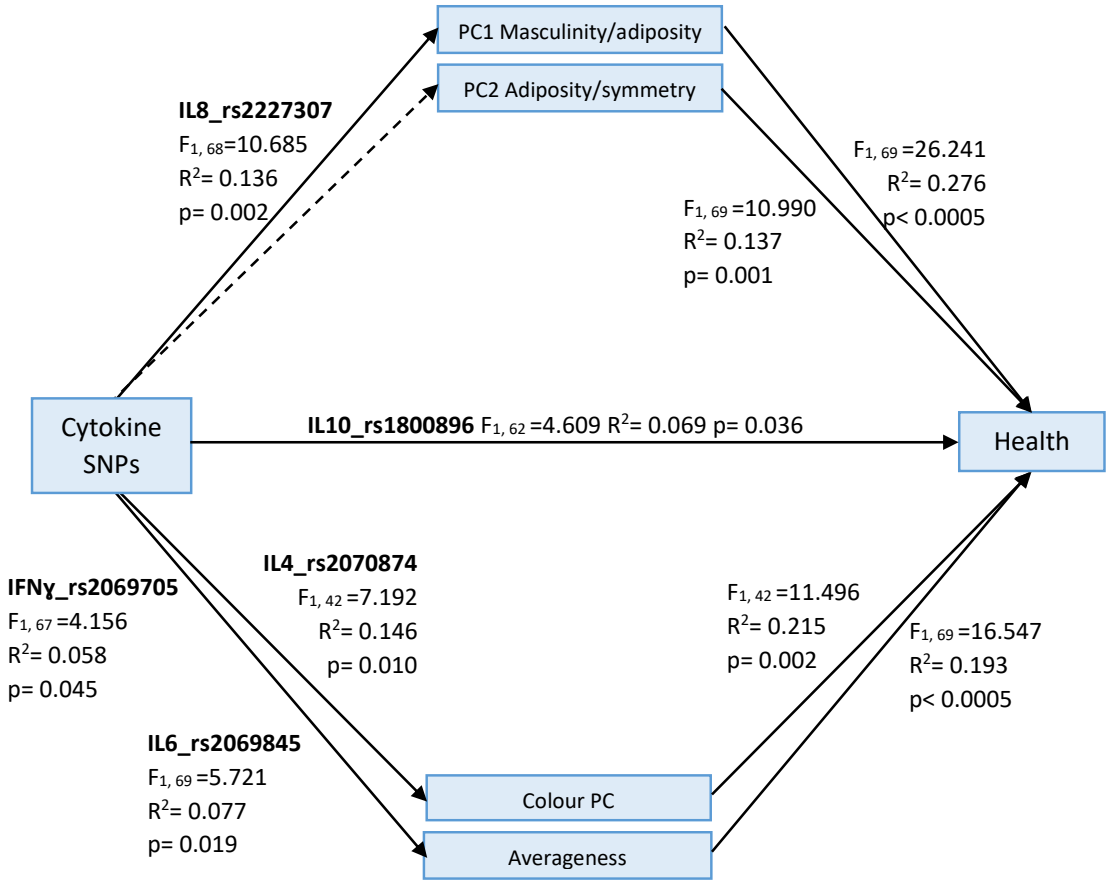


Figure 2.8 *Overview of significant linear regression relationships between cytokine related SNPs, apparent health and the facial cues that influence apparent health*

2.3.4. Experiment two analysis

Linear regressions

Separate linear regressions were performed with the nine SNPs as the independent variable and seven structural PCs as the dependant variable to assess the relationship between genetic variation in cytokine SNPs and facial shape (Table 2.8).

Three SNPs were significantly associated with facial shape components. SNP rs10499563 (C/T) in *IL-6* was significantly associated with PC 1 ($F_{1, 66}$=4.639, R^2=0.066, p=0.035). Higher PC 1 values are attributed to the C allele. The SNP in *IL-10* (rs1800896) was highly significantly associated with PC 3 ($F_{1, 62}$=7.661, R^2=0.110, p=0.007) with the C allele giving rise to higher PC 3 values and the T allele resulting in lower PC 3 values. Lastly, a highly

significant relationship was found between rs2227307 (a T/G polymorphism found in *IL-8*) and PC 7 ($F_{1, 68}$=7.305, R^2=0.097, p=0.009); the T allele is related to higher PC 7 values.

Table 2.8. *Observed F statistics (the top number) and R^2 values (the bottom number) after separate linear regressions between SNP and facial structural principal components*

dbSNP	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
IL-2 **rs2069771**	$F_{1,62}$ =2.301 R^2= 0.036	$F_{1,62}$ =0.340 R^2= 0.005	$F_{1,62}$ =0.000 R^2= 0.000	$F_{1,62}$ =1.205 R^2= 0.019	$F_{1,62}$=1.025 R^2= 0.016	$F_{1,62}$ =1.162 R^2=0.018	$F_{1,62}$ =0.021 R^2=0.000
IL-4 **rs2070874**	$F_{1,69}$ =0.135 R^2= 0.002	$F_{1,69}$ =0.727 R^2= 0.010	$F_{1,69}$ =2.714 R^2= 0.038	$F_{1,69}$ =0.207 R^2= 0.003	$F_{1,69}$ =0.004 R^2= 0.000	$F_{1,69}$ =3.652 R^2=0.050	$F_{1,69}$ =0.769 R^2=0.011
IL-6 **rs10499563**	**$F_{1,66}$ =4.639*** **R^2= 0.066**	$F_{1,66}$ =0.001 R^2= 0.000	$F_{1,66}$ =0.104 R^2= 0.002	$F_{1,66}$ =0.055 R^2= 0.001	$F_{1,66}$ =0.039 R^2= 0.001	$F_{1,66}$ =0.778 R^2=0.012	$F_{1,66}$ =0.080 R^2=0.001
IL-6 **rs2069845**	$F_{1,69}$ =0.107 R^2= 0.002	$F_{1,69}$ =0.430 R^2= 0.006	$F_{1,69}$ =0.739 R^2= 0.011	$F_{1,69}$ =0.222 R^2= 0.003	$F_{1,69}$ =0.137 R^2= 0.002	$F_{1,69}$ =3.657 R^2= 0.050	$F_{1,69}$ =1.567 R^2=0.022
IL-8 **rs2227307**	$F_{1,68}$ =0.069 R^2= 0.001	$F_{1,68}$ =0.044 R^2= 0.001	$F_{1,68}$ =1.361 R^2= 0.20	$F_{1,68}$ =0.878 R^2= 0.013	$F_{1,68}$ =0.003 R^2= 0.000	$F_{1,68}$ =0.693 R^2=0.010	**$F_{1,68}$ =7.305**** **R^2=0.097**
IL-10 **rs1800896**	$F_{1,62}$ =0.871 R^2= 0.014	$F_{1,62}$ =2.150 R^2= 0.034	**$F_{1,62}$ =7.661**** **R^2= 0.110**	$F_{1,62}$ =0.823 R^2= 0.013	$F_{1,62}$ =0.000 R^2= 0.000	$F_{1,62}$ =0.003 R^2=0.000	$F_{1,62}$ =0.040 R^2=0.001
IFN-ɣ **rs2069705**	$F_{1,67}$ =0.528 R^2= 0.008	$F_{1,67}$ =2.810 R^2=0.040	$F_{1,67}$ =0.096 R^2= 0.001	$F_{1,67}$ =0.494 R^2= 0.007	$F_{1,41}$ =1.257 R^2= 0.018	$F_{1,67}$ =0.004 R^2=0.000	$F_{1,67}$ =0.001 R^2=0.000
CSF2 **rs25882**	$F_{1,68}$ =0.032 R^2= 0.000	$F_{1,68}$ =3.103 R^2= 0.044	$F_{1,68}$ =0.107 R^2= 0.002	$F_{1,68}$ =0.007 R^2= 0.000	$F_{1,68}$ =0.343 R^2=0.005	$F_{1,68}$ =0.021 R^2=0.000	$F_{1,68}$ =1.599 R^2=0.023
CSF2 **rs721121**	$F_{1,69}$ =0.265 R^2= 0.004	$F_{1,69}$ =0.242 R^2= 0.003	$F_{1,69}$ =0.125 R^2= 0.002	$F_{1,69}$ =0.183 R^2= 0.003	$F_{1,69}$ =0.812 R^2=0.012	$F_{1,69}$ =0.267 R^2=0.004	$F_{1,69}$ =0.140 R^2=0.002

*p<0.05, **p<0.01, ***p<0.001

Characterising significant structural PCs

Three structural PCs revealed to have significant associations with cytokine SNPs; PC 1, PC 3 and PC 7 (Table 2.8). To further understand these results, each of the structural PCs were characterised by observing the average faces for the low and high values of the PCs (Figure 2.9 A, B and C). We also investigated the significant associations these structural PCs have with facial judgements obtained from experiment one.

Structural PC 1 explains 32% of the facial variance and it was significantly and positively correlated with perceptual PC 2 (+symmetry/-adiposity; r= 0.317, p=0.007) and health perception (r=0.237, p=0.046). Faces with lower structural PC 1 values do appear to be heavier and more symmetrical compared to faces with higher PC 1 values (Figure 2.9 A).

Faces with lower PC 3 values also appear to have heavier faces (Figure 2.9 B). Structural PC 3 explains 12% of facial variance and is also positively associated with the 2 +symmetry/-adiposity component (r=0.330, p=0.005). Structural PC 7 is the only structural PC that is significantly associated with the +masculinity/+adiposity component (r=-0.322, p=0.006). This significant correlation is negative and can be observed in the lower average face for structural PC 7 that appears heavier and more masculine than the higher structural PC 7 faces (Figure 2.9 C). PC 7 explains 3% of the facial variance. Interestingly, the SNPs were only significantly associated with structural components that were related to health perception or perceptual cues that influence health appearance (only one structural component, PC 5, that was significantly related to +symmetry/-adiposity, did not have a significant relationship with a cytokine SNP).

Unfortunately, no results were significant after a Bonferroni corrected alpha of p=0.000463.

Figure 2.9. *Average faces of 10 participants with the lowest (images on the left) and 10 participants with the highest (images on the right) values for the respective variables (Structural PC 1 (A), PC3 (B) and PC7 (C)). Frontal and profile images are presented for each average face*

2.4 Discussion

The aim of this study was to assess the association between cytokine related SNPs and facial appearance in African men. The genetic focus was centred on nine SNPs associated with seven cytokine genes (a SNP in *IL-2, IL-4, IL-8, IL-10* and *IFN-γ* and two SNPs in *IL-6*

and *GM-CSF*). Facial appearance was measured as different variables in experiment one and experiment two. In experiment one we assessed the relationship that the SNPs have with health perception as well as the facial cues that have been previously shown to influence health perception (measured skin colour and perceived masculinity, adiposity, symmetry and averageness). In experiment two, we investigated the association between these SNPs and facial structure explained by structural components of the face gathered from a PCA of x and y coordinates from a total of 71 points that were plotted on facial images. We did in fact find a number of significant associations between SNPs in cytokine genes and facial appearance. As well as more intricate findings between health perception and the facial cues that influence health perception.

IL-4 SNP and skin colour

An exclusive significant relationship was observed between rs2070874 in the promoter of *IL-4* (will now be referred to as the IL-4 SNP) and the skin colour PC. The *IL-4* SNP was only associated with skin colour and no structural components of the face. IL-4 has in fact been shown in previous studies to play a role in skin homeostasis (Elbe-Burger *et al.* 2002; Sehra *et al.* 2010) and skin colour changes that occur during an immune response (Choi *et al.* 2013). IL-4 promotes the humoral and inhibits the cellular immune response (Swain *et al.* 1990; Gautam *et al.* 1992). The cytokine plays an important role in asthma (Broide *et al.* 1992; Shirakawa *et al.* 2000) and is responsible for the production of the IgE antibodies that cause allergies (Finkelman *et al.* 1988).

The T-allele of this SNP is significantly associated with the skin colour that appears less healthy (darker skin with a less yellow and red skin tone). There are 3 possible explanations for why we observed this relationship. Firstly, the T-allele has been previously shown to increase *IL-4* expression and protein production (Nakashima *et al.* 2002; Gonzales *et al.* 2010; Anovazzi *et al.* 2017). Initially, this may seem contradictory but, due to the role that IL-4 plays to promote allergies, asthma and inflammation, higher levels of IL-4 results in hyperactivity of the immune system (Choi and Reiser 1998) which is in fact unhealthy.

Secondly, IL-4 has been shown in previous studies to play a role in skin colour through inhibition of melanogenesis in normal human melanocytes that were cultured from Japanese, Chinese and Korean ethnic backgrounds (melanogenesis is the production of

melanin, a pigment that is responsible for a darker skin tone; Choi *et al.* 2013). During an immune response or an allergic reaction (after the release of IL-4) Caucasian and Asian skin increases in redness and hypopigmentation (lighter skin) occurs. This is not in agreement with the current study as we observed that the allele affiliated with higher IL-4 levels is associated with darker skin that is not as red in colour. This discrepancy can be explained through the difference in skin colour changes, due to an immune response, that occurs between Africans, Caucasians and Asians (Taylor 2002). For example Choi *et al.* (2013) noted that the effects of cytokines on melanocytes varies, according to the ethnicity origin of the melanocyte. The same cytokine concentrations were introduced to cultured melanocytes from different ethnicities. Melanogenesis was inhibited in Chinese/Korean/Japanese melanocytes, the viability of African-American melanocytes was compromised and not much change was observed in the Caucasian melanocytes (a much higher concentration of these cytokines was added to the Caucasian melanocytes before their viability was compromised; Choi *et al.* 2013). Hyperpigmentation (darker skin) that occurs due to an immune response (for example “post-inflammatory hyperpigmentation”) is also much more common in darker skinned individuals as opposed to lighter skinned or Caucasian individuals (Davis and Callender 2010). It is still unclear why people of different ethnicities experience different changes in skin colour due to an immune response (Taylor 2002).

Thirdly, a less yellow skin tone that is observed with the T-allele could be explained through IL-4’s vital role in asthma and allergies. Individuals that suffer from asthma and allergies have increased levels of IL-4 which leads to an increase in free radicals and other ROS that cause oxidative stress (Dworski 2000; Sahiner *et al.* 2011; Qu *et al.* 2017). Anti-oxidants such as B-carotene (a type of carotenoid) are used in the body to neutralise these free radicals and other ROS (Johnson 2002). The T-allele could be related in this study to a less yellower skin tone as carotenoids in the body (responsible for giving skin its yellow colour (Stephen *et al.* 2011; Whitehead *et al.* 2012)) are used to neutralise ROS (Johnson 2002) caused by asthma and allergies, this leads to less carotenoid deposition in the skin cells. Individuals that have higher carotenoid levels, specifically B-carotene, have in fact been found to have fewer reported respiratory infections (Van Der Horst-Graat *et al.* 2007).

IL-8 SNP and the +masculinity/+adiposity component

One of the most significant genetic relationships we found with facial appearance was with the SNP we investigated in intron 1 of *IL-8* (rs2227307; T/G), the only chemokine that was investigated in this study. Chemokines induce inflammation in the innate immunity and recruit immune cells to the site of infection (Luster 1998). The T allele of the *IL-8* SNP is the variant within the literature that has been associated with lower health. The T allele has shown to be a risk factor for certain diseases such as chronic periodontitis (Zhang *et al.* 2014) and with respiratory health issues such as idiopathic pulmonary fibrosis (Ahn *et al.* 2011) and lung function decline in chronic obstructive pulmonary disease (Córdoba-Lanús *et al.* 2015).

The relationship between the *IL-8* SNP and the +masculinity/+adiposity component was revealed in both experiment one and experiment two. The T-allele of the *IL-8* SNP was associated with lower +masculinity/+adiposity component values which were strongly correlated with lower health ratings. IL-8 is the only chemokine we explored in this study and the *IL-8* SNP was the only SNP in this study that was significantly associated directly with the +masculinity/+adiposity component in experiment one. In experiment two, the *IL-8* SNP revealed an exclusive relationship with the only structural PC (PC 7) that was significantly correlated with the +masculinity/+adiposity component.

The +masculinity/+adiposity component is more representative of the under-normal weight individuals in this sample cohort hence why adiposity in this component increases with health perception. This positive relationship between adiposity and health in lower weight individuals is in accordance with the literature as individuals who are underweight have been shown to have low immune functioning and poorer health compared to normal weight individuals (Ritz and Gardner 2006). IL-8 is an important pro-inflammatory cytokine and inflammation and weight have in fact been closely linked in the literature (Plata-Salaman and Borkoski 1993; Mora and Pessin 2002; Lyon *et al.* 2003). However in the current study the *IL-8* SNP is mostly associated with adiposity in under-normal weight individuals. The *IL-8* SNP may in fact be related with muscularity. Previous work proposed that the +masculinity/+adiposity component indicates muscularity (a measure of strength) in the face (Phalane *et al.* 2017). Higher testosterone levels increase muscularity and body weight as well as facial masculinity (Bhasin 2003; Penton-Voak and Chen 2004).

Therefore, in the underweight-normal weight men in this study, muscle and not fat likely plays an important role in this component (Phalane *et al.* 2017).

The relationship that the +masculinity/+adiposity component has with the *IL-8* SNP and health perception may support the ICHH which states that testosterone mediated traits (such as facial masculinity/muscularity (Penton-Voak and Chen 2004)) could indicate health. Testosterone is an immunosuppressant, therefore only males that have a superior ability to fight off infections, can afford to have higher testosterone levels (Folstad and Karter 1992). Facial masculinity has been associated with some measures of immunity such as self-reported respiratory infections, response to hepatitis B vaccinations and cytokine response (Thornhill and Gangestad 2006; Rantala *et al.* 2013a; Phalane *et al.* 2017). However, other measures of the immune system such as self-reported anti-biotic use and HLA heterozygosity have no significant relationship with facial masculinity (Lie *et al.* 2008; Boothroyd *et al.* 2013). In the current study, masculinity was only related to a chemokine SNP and no other cytokine polymorphisms. Therefore masculinity/adiposity (likely muscularity) may indicate certain aspects of the immune system but not others.

IL-10 and health perception

IL-10 is a strong anti-inflammatory cytokine primarily involved in the humoral immune response. Rs1800896 is a SNP found in the promoter of *IL-10* that we investigated in this study. This SNP forms part of a haplotype group with two other SNPs (GCC) that have been associated with increased production in vivo (Turner *et al.* 1997), stronger transcriptional activity (Crawley *et al.* 1999) and higher mRNA expression (Suarez *et al.* 2003) of *IL-10*. Analysing rs1800896 independently from the other two SNPs still revealed significant results with *IL-10* expression; the c allele is significantly associated with higher mRNA expression, serum protein levels and ex vivo levels of IL-10. (Suarez *et al.* 2003).

The *IL-10* SNP was the only SNP to be directly associated with overall health appearance; the C allele was significantly associated with lower health perception. It is unclear what facial component mediates this relationship as the *IL-10* SNP was not directly associated with any other perceptual components. Interestingly in experiment two, the C allele of the *IL-10* SNP revealed the opposite relationship with health appearance, although it was an indirect relationship. The C-allele of the *IL-10* SNP had a significant association with higher structural PC 3 values (a much stronger association was found with PC 3 than with health

perception in experiment one). PC 3 explains a relatively high percentage of the variance in the face (11.88%) and is highly significantly and positively correlated with the +symmetry/–adiposity component, which is positively correlated with health perception. Although the *IL-10* SNP influences facial appearance, there are discrepancies between the two experiments as to how it influences health perception specifically. This could be due to the fact that the *IL-10* SNP may influence other factors in facial appearance that were not explored in this study.

IL-6 and facial structure

The SNP found in the promoter of *IL-6* (rs10499563) had no significant relationships in experiment one however a significant relationship was discovered in experiment two with the structural component that explained the highest variance in the face (PC 1 that explains 32% of the variance). PC 1 was highly significantly and positively correlated with the +symmetry/–adiposity component (which is positively correlated with health perception). PC 1 is also the only structural component that was directly and significantly associated with health perception.

IL-6 is an important pro-inflammatory cytokine that plays an important role in the entire immune response. The T allele for this SNP is found on an enzyme recognition site and is associated with an increase in *IL-6* expression (Joffe *et al.* 2014). The T allele in this study is significantly associated with higher PC 1 values that are positively correlated with higher +symmetry/–adiposity and health perceptions.

IL-6 and averageness

There was another SNP that was investigated in the *IL-6* gene. Rs1554606 is a SNP found in intron 4 of the *IL-6* gene and was slightly significantly associated with facial averageness. The SNP revealed no other significant relationship in this study. And averageness was not significantly correlated with any structural components. The G allele of the SNP in *IL-6* has been shown to alter enzyme recognition sites. It may be the more detrimental allele that is associated with obesity and aversive symptoms in cancer patients that result due to an increase of pro-inflammatory cytokines (Joffe *et al.* 2014; Doong *et al.* 2015; Todendi *et al.* 2015). The G allele in this study is in fact significantly associated with lower perceived averageness which is significantly correlated to lower health perception.

IFN-γ and averageness

Similar to the *IL-6* SNP found in intron 4, the SNP that we investigated in *IFN-γ* (rs2069705) was significantly associated with averageness. Like *IL-6, IFN-γ* is also a pro-inflammatory cytokine. The A allele in the A/G SNP is associated with higher averageness perceptual scores. *IFN-γ* is primarily involved in the cellular immune response pathway (Ijzermans and Marquet 1989; Samuel 2001) and is the only cellular immune cytokine in this study that has a significant relationship with facial appearance (although the relationship is not very strong). This particular SNP has been suggested to be associated with differential levels of *IFN-γ* although its effect on actual expression has not been studied directly (Kim *et al*. 2010).

2.5 Conclusions

We found significant associations between 6 cytokine-associated SNPs and facial appearance in African men: rs2070874 in *IL-4* was significantly associated with skin colour, rs2227307 in *IL-8* with +masculinity/+adiposity, rs2069845 in *IL-6* and rs2069705 in *IFN-γ* with averageness and rs10499563 in *IL-6* and rs1800896 in *IL-10* with structural components 1 and 3 respectively that are significantly associated with +symmetry/–adiposity. Only one SNP (rs1800896 in the *IL-10* gene) was directly associated with perceived health. Interestingly, all SNPs that were associated with cytokines that play a primary role in the humoral immune response (IL-4,-6 and-10) had significant relationships with facial appearance. Only one SNP in the cellular immune response (*IFN-γ*) and innate immune response (*IL-8*) had significant relationships with facial appearance. However, it is to be noted that although cytokines have primary functions within the immune system, most cytokines play various and multiple roles in the immune response and in immune homeostasis. The relationship between facial appearance and immunity is expectantly complicated and it appears that different aspects of the immune system may be related to different aspects of facial appearance.

It is very important to note that these results should be interpreted with caution given the small sample size. A major concern for this study is that there is no longer significance after Bonferroni correction. This is not surprising as Bonferroni correction is recognised as a very stringent method of correction for multiple testing (Armstrong 2014). It is still very important to conduct further research with better statistical power (possibly through

increasing the sample sizes or number of SNPs) to determine whether the associations in this study hold. Although my results are non-significant after correcting for multiple testing, it is still very important to report. Non-significant findings can be just as insightful as significant results. Unfortunately non-significant findings are not often published and this leads to publication bias within the literature.

The findings in the current study are however, still pertinent and worth reporting given that this is the first study to find associations between facial appearance and cytokine-associated genetic variation. The face is a complex polygenic trait that is influenced by environmental factors and finding a single SNP that significantly influences facial appearance is very fascinating. What is even more extraordinary is the discovery of multiple cytokine related SNPs that have a significant relationship with facial appearance. The percentage variance in the face explained by the SNPs is small, as is expected in a highly polygenic trait.

This study has provided possible further evidence of the complicated relationships between facial appearance and health (specifically immunity) in men (Roberts *et al.* 2005; Lie *et al.* 2008; Rantala *et al.* 2013a; Phalane *et al.* 2017). Findings may also expand the genetic knowledge we already have between facial appearance and health that we've obtained from HLA studies.

Chapter 3

This study focussed on assessing SNPs within multiple cytokines that play a role in various aspects of the immune response as well as general immune homeostasis. Different cytokines were significantly associated with different aspects of an individual's facial appearance. Therefore various facial cues that influence health appearance could be related to different immune responses or other aspects of actual health. The complicated relationship between health and facial appearance should prompt more studies to assess the relationship between facial appearance and direct measures of immunity that represent various aspects of the immune response. To date, only response to hepatitis B vaccinations and cytokine response have been investigated. We found that there are strong and intricate relationships amongst the perceptual judgments. This should encourage future studies to assess facial health cues together as opposed to studying them separately.

The current study found significant associations between *IL-4, -6, -8, -10* and *IFN- γ* SNPs with facial appearance. We did not investigate how these SNPs influence cytokine activity, if they directly influence immunity and health or if they are in LD with another functional SNP. Future studies could investigate the mechanisms that may mediate these associations between the SNPs and facial appearance. Through these studies we could gain a more global understanding between health, specifically immunity, and facial appearance.

A few challenges were highlighted during the literature search for the candidate SNPs for this study. In a candidate gene study, it is very important which candidates are chosen as it will aid in the validity of the final results. I chose cytokines as candidate genes as they are intimately involved in the entire immune response as well as homeostasis of the immune system (Vilček 2003). Therefore cytokines can be a good representation of an individual's immunity and overall health. A recently published paper has also found that cytokine activity levels are associated with facial appearance (Phalane *et al.* 2017). Choosing the SNPs within the cytokine genes to be studied was done as meticulously as possible. The final SNPs chosen were all related in some manner to cytokine activity or health and they had a MAF in the African populations >25%. Certain SNPs that could be

considered as more important or relevant as they had been studied more extensively, were not chosen for this experiment due to very low MAF that they had in the African populations. Similarly, some of the SNPs that had a high enough MAF in African populations had low MAF in other populations (Caucasian or Asian populations), therefore they were not studied as extensively and there were a lack of studies surrounding the SNPs. This issue further highlights the importance of this study conducted in an African population, a population that has been relatively under-represented.

The SNPs chosen for this study spread across different cytokine genes found on different chromosomes that play numerous important roles in the immune response. We assessed polymorphisms in a variety of cytokine genes that primarily function in the humoral (*IL-4,* *IL-6,* and IL-10), the cellular (*IL-2* and *IFN-γ*) and innate immune responses (*IL-8* and *GM-CSF*). I was however limited in the number of SNPs I could choose for this study. The various significant results from the current study should lead to the investigation for more candidate SNPs within the current genes as well as candidate SNPs in other cytokine genes to be assessed in future facial appearance studies. Increasing the number of SNPs in the study could increase the statistical power and could even reveal significant results after correction for multiple testing, which I was unfortunately not able to do in this study.

As mentioned earlier, the biggest limitation to the current study is the sample size. We however, took several measures to maximise the power of the analyses given the sample size. First, we recruited a fairly homogenous group of participants (Black South African men aged between 18 and 25). Second, we selected candidate SNPs with a high minor allele frequency (>25%) in the African populations, thereby increasing the power of the analyses (Hong and Park 2012). Third, we minimised the number of independent tests by reducing the number of facial appearance variables through principal components analysis. One of the reasons for the small sample in the current study was because of the unwillingness of the participants to give blood samples. For future studies DNA will be obtained through a more non-invasive measure such as saliva. This would encourage participants and increase the sample size.

It would also be interesting to assess the relationships between cytokine related SNPs and facial appearance in different populations from different ethnic backgrounds as well as different genders since immune responses and facial cues that influence health perception

differ in genders and ethnicities. Potential environmental influences (smoking, sun exposure, living conditions etc.) were not controlled for in this study. In a larger sample, more demographic information should be collected and considered during analysis. Our research group has also moved to 3D imaging, which will be beneficial in future studies as 3D facial images will be able to capture more variation within the face that this study might have missed.

Facial appearance is a very complex polygenic trait that is also influenced by environmental factors. We were still able to find marginal significant relationships between cytokine SNPs and facial appearance. The SNPs within these cytokines (or correlated SNPs) may directly influence facial development and appearance or they may influence how facial appearance adapts and develops in response to the environment. This study is the first to find a significant association between facial appearance and cytokine related genes. It is also one of the very few studies assessing health and facial appearance within an African population.

This study may be beneficial in various fields of research such as forensics and evolutionary studies. There is on-going research in the forensics field to find the genes that influence facial appearance so that unknown victims or suspects may be identified from DNA left at a crime scene. Our findings may expand the scope of potential candidate genes which influence variation within the face. We also show in this study, that facial traits which influence attractiveness are significantly associated with immune based genes such as cytokines. These results could lead to future studies that would provide further evidence and support for the evolutionary “good-genes” hypothesis which states that attractive traits may indicate biological quality.

This research is extremely novel and it could lay the foundation for many future studies to better understand the genes that influence facial appearance and to better understand the relationship between facial appearance and health.

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