Determining the link between facial appearance and immunity in an African population

by

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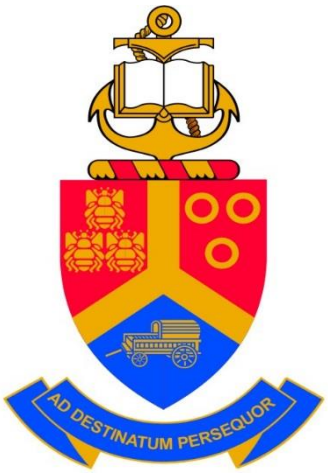

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Declaration

I, Khutso Gemina Phalane, hereby declare that the work contained in this thesis is my own original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature: …… Date: …June 2017……

Preface

Facial appearance is thought to indicate immunity in humans, but very few studies have tested this relationship directly. The aim of the work in chapter 2 was to test the relationship between direct measures of immunity, apparent facial health and attractiveness, and facial cues in African men. In chapter 2 we show that men with a stronger cytokine response are considered significantly more attractive and healthy. Men with more masculine, heavier facial features (i.e. muscular appearance) have a significantly higher cytokine response and appear significantly healthier and more attractive, while men with a yellower, lighter, “carotenoid” skin tone, have a marginally higher immune response and are also considered significantly healthier and attractive. In contrast, more symmetrical, skinnier looking men appeared more attractive and healthier, but did not have a stronger cytokine response. These findings shed new light on the “androgen-mediated” traits proposed by the immunecompetence handicap hypothesis (ICHH). Finally, we build on previous evidence to show that men’s facial features do indeed reveal aspects of immunity, even better than more traditional measures of health, such as body mass index (BMI).

The major histocompatibility complex (MHC) is one of the best studied genetic mating systems which is referred to as the Human Leucocyte Antigen (HLA) in humans and is an indirect measure of immunity. The aim of the work in chapter 3 was to test the relationship between two HLA based mating preferences (preference for HLA heterozygosity and a preference for common HLA alleles), facial cues (*e.g.* masculinity, symmetry) and overall facial appearance (attractiveness and health) in African men. We show that an HLA-associated SNP (rs2524079 in the HLA-C region) which has been linked to lymphocyte count is positively associated with facial appearance in an African population. Our results also show that the relative contribution of different aspects of immunity might differ between different populations. While HLA heterozygosity has been positively associated with facial attractiveness in British and Australian men, specific common HLA-associated alleles seem to play a larger role in African men.

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Note to the reader

Throughout the experimental chapters in this thesis, I have used the pronoun ‘we’ instead of ‘I’. This work is my own in terms of hypotheses, analyses and conclusions; however, the Facial Morphology Research Laboratory is a collaborative environment with other members assisting in the recruiting of participants and the collection of samples. The plural pronoun reflects the fact that, if published, the following experiments would carry multiple authorship and is used in keeping with intellectual honesty.

List of abbreviations

AIDS	:	Acquired Immune Deficiency Syndrome
ANOVA	:	Analysis of Variance
BMI	:	Body mass index
BP	:	Blood Pressure
CRP	:	C- reactive protein
CTL	:	Cytotoxic T Lymphocytes
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked Immunosorbent Assay
FA	:	Fluctuating asymmetry
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
HWE	:	Hardy-Weinberg Equilibrium
HLA	:	Human Leukocyte Antigen
IFN-y	:	Interferon gamma (IFN-y)
ICHH	:	Immunocompetence Handicap Hypothesis
IL	:	Interleukin
LPS	:	Lipopolysaccharides
MAF	:	Minor Allele Frequencies
MHC	:	Major Histocompatibility Complex
NK	:	Natural Killer
PBMC	:	Peripheral blood mononuclear cell
PBS	:	Phosphate-buffered saline
PCA	:	Principal component Analyses
PSA	:	Penicillin-Streptomycin-Amphotericin B
RPMI	:	Roswell Park Memorial Institute medium
SD	:	Standard Deviation
SNP	:	Single Nucleotide Polymorphism
SSSM	:	Standard social science model
Th	:	T helper
TNF	:	Tumor necrosis factor
UV	:	Ultraviolet
WHO	:	World Health Organisation

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Chapter 1: Literature review

Facial appearance, health and Immunity

1. Introduction

Physical appearance plays an important role in mate choice (1-4). Most non-human species rely on external traits, such as size, shape and colour of adornments to attract mates (3). For example, female *Eumeces laticeps* (broad-headed skinks) prefer larger males as potential mates as compared to males with smaller bodies (4). Various evolutionary hypotheses have developed to explain why females prefer certain external traits in potential male mates. According to the "good genes" hypothesis, females choose their mates based on traits that indicate high genetic quality, especially in terms of disease resistance (5, 6). The handicap principle proposes that females choose mates with a handicap, which serves as an "honest signal "of quality, as the ability of the individual to sustain the handicap indicates the ability of the individual to withstand environmental pressures (7).

The immunocompetence handicap hypothesis builds on the previous hypotheses, proposing that testosterone in humans causes exaggerated secondary sexual cues in males but is also an immunosuppressant (8). Thus exaggerated secondary sexual cues indicate genetic quality in that the male could contend with the immunosuppressive effects of androgens. Kimball and Ligon (9) however, argue that there are inconsistencies with the immunocompetence handicap hypothesis in that not all secondary sexual traits are testosterone-dependent but are controlled by the lack of oestrogen, increased luteinizing hormone or by non-hormonal factors (9, 10).

1.1. Facial appearance

Facial appearance plays an important role in human mate choice (1, 2, 11-14). Physically attractive men have been reported to have more female sexual partners and more offspring (15-17). In women, physical attractiveness is positively associated with reproductive success. Jokela *et al.* (18) showed that attractive women had 16% and very attractive women 6% more children than their less

attractive counterparts. Facial attractiveness has also been positively associated with economic mobility (19), health (20-22), fertility (23-25) and longevity (26).

It was traditionally believed that standards of attractiveness are learned gradually through exposure to culturally presented ideals and that different cultures would vary dramatically in what they perceive to be attractive (27-31). According to the standard social science model (SSSM) which was first introduced in 1992 by Tooby and Cosmides, humans are born a blank slate and that culture/socialization determines behaviour and standards (32). This would mean that attractiveness is arbitrary and that the perception of what is attractive would change depending on the geographic location and era (33). Work on cross-cultural agreement in attractiveness preferences strongly suggests that attractiveness ideals are not merely absorbed by cultural influences, but that there is something universal about attractive (and unattractive) faces that is recognized both across individuals and cultures (34).

In a meta-analysis, Langlois *et al.* (35) showed that raters agree about who is and is not attractive both within and across cultures in both adults and children. Furthermore, infants and adults agree on which faces are attractive and which are not; studies conducted on infants (including newborn infants) show that they prefer to spend more time looking at faces which were rated as attractive by adults as compared to faces that were rated unattractive (29, 36, 37). These infants have had limited or no cultural influences yet. Therefore, this adult-infant agreement in attractiveness preferences is in line with the findings that perceptions of attractiveness are universal and not merely absorbed through cultural influences.

1.1.1. Associations between Facial Attractiveness and Health

Facial attractiveness is positively associated with perceived health, in that more attractive individuals are also considered healthier looking (20, 22, 24, 38, 39). This association between perceived health and attractiveness could be ascribed to the “attractiveness halo”— the tendency of people to rate attractive individuals more favourably than less attractive individuals (40) — or could be ascribed to a real relationship between attractiveness and health. It is therefore also important to determine the relationship between actual health and attractiveness.

Some previous studies reported significant associations between facial attractiveness and measures of actual health. Actual health measures vary in quality from indirect measures of health, such as self-reported health and longevity, to direct measures of health, such as the antibody response after vaccination. Hume and Montgomerie (20) found that attractive women (but not men) report significantly fewer severe diseases during their lifetime. Shackelford and Larsen (41) found that increased facial attractiveness significantly correlated with some common physical illness symptoms (*e.g.* less runny or stuffy noses and less sore throats) and increased cardiovascular recovery time after exercise in men and less headaches in women. Kalick *et al.* (21) did not find a significant correlation between late adolescent facial attractiveness and health scores based on detailed medical histories. Similarly, a study conducted by Thornhill and Gangestad (42) also did not find a significant association between facial attractiveness and self-reported use of antibiotics or the number and duration of respiratory and stomach infections in the last three years of their study. As can be seen above, studies using self-reported measures of health or health histories found an inconsistent relationship between attractiveness and actual health.

More recent studies tested the association between facial attractiveness and immunity more directly. Several studies focused on the Major Histocompatibility Complex (MHC) genes, or Human Leukocyte Antigen (HLA) genes in humans. Heterozygosity at HLA loci increase the number of antigen peptides that can be displayed to the immune system and therefore improves the individual's ability to resist a broader array of pathogens (46). Several studies have found a positive association between male facial attractiveness and HLA heterozygosity (43-45). Roberts *et al.* (45) found that young men who are HLA heterozygous are considered more attractive than their HLA homozygous counterparts. Lie *et al.* (43) reported that HLA heterozygosity also positively predicted male attractiveness. In contrast, studies conducted in African (46) and Caucasian women (43, 44) did not find a significant association between HLA heterozygosity and facial attractiveness. Thornhill *et al.* (47) did not find any relationship between HLA heterozygosity and facial attractiveness judgments in men and women faces, however there where great age differences within their study population ranging between 18-54 years for men and

17-44 years for women. The facial attractiveness judgments could therefore have been confounded by age. Perhaps the most direct evidence comes from a study by Rantala *et al.* (48), which reported a significant relationship between a direct measure of immune response (Hepatitis B antibody response) and facial attractiveness in men. They found that attractive men produced significantly higher antibody levels after Hepatitis B vaccination than less attractive men (48). In contrast, facially attractive women did not produce significantly higher Hepatitis B antibody levels after vaccination compared to less attractive women (49). Foo *et al.* (50) did not find a significant association between immunity (bacterial killing capacity, overall bacterial immunity, bacterial suppression capacity and lysozyme activity) and facial appearance in both Caucasian men and women. Therefore studies using direct measures of immunity generally found positive relationships between attractiveness and immunity in men, but not in women.

Facial attractiveness is also associated with fertility (23-25). Hill and Hurtado (23) found that facially attractive Ache Indian women are 1.2 times more fertile (fertility determined by the number of offspring) than their average attractive counterparts (with age-controlled groups). Facially attractive women have been shown to have higher late follicular oestrogen levels than less attractive woman (24). When the morphology and motility of male sperm was assessed by Soler *et al.* (25) it was found that attractive men had better sperm quality as compared to less attractive men.

1.2. Facial Cues that play a role in Attractiveness

Although it is fairly easy to judge whether a face is attractive or unattractive, it is difficult to articulate the specific features that determine this attraction (39). Several facial traits have been shown to influence attractiveness, namely symmetry (12, 51-57), averageness (how closely the face resembles the majority of other faces in the population; (12, 22, 29, 58-61), sexual dimorphism (masculinity/ femininity; (22, 54, 62-66), skin colour (67-72) and facial adiposity (or facial fatness; (73-77). We will discuss each facial cue in turn.

1.2.1. Facial Symmetry

Symmetry is defined as beauty of form arising from balanced proportions (78). In order to be symmetrical, the one side of the face or object should be a reflection of the other side of the face or object (12, 79, 80). Individuals are exposed to different environmental pressures and stressful conditions which influence the development of their morphology. An ability to successfully maintain one's morphology under the prevailing conditions results in more symmetrical faces (81-83).The optimal developmental outcome is symmetry. Fluctuating asymmetry (FA) is a useful measure of developmental stability because we know that the optimal developmental outcome is symmetry (84, 85). Furthermore, FA is thought to be influenced by both genetic (*e.g.* inbreeding, mutation and homozygosity and environmental (*e.g.* nutrient intake and parasite load) factors on individual development (81, 82, 86). Therefore, individuals who are able to develop symmetrically under harsh environmental conditions are proposed to have greater genetic quality (11, 81, 82)**.**

Directional asymmetry is another form of asymmetry which does not indicate developmental stability (84, 87). It is characterised by a symmetry distribution which is significantly bias towards larger traits either on the left or the right side of the face (88). A study by Simmons *et al.* (89) measured both fluctuating and directional asymmetry in a population of mixed ethnicities (*e.g.* Caucasian, Asian, Eurasian, African, Australian Aboriginal, New Zealand Maori, Hispanic, and Lebanese) and showed that directional asymmetry did not affect attractiveness judgements. The results in the Simmons *et al.* (89) study suggest that people focus more on aspects of facial asymmetry that may be revealing of developmental instability and not necessarily directional asymmetry. More symmetrical faces could provide both direct (*e.g.* by avoiding contagion) and indirect benefits (*e.g*. by providing healthy genes for offspring) to the perceiver (90).

1.2.2. Associations between Facial Symmetry and Attractiveness

Numerous studies have demonstrated that facial attractiveness is positively associated with facial symmetry *e.g*., (12, 50, 54, 56, 79) and bodily symmetry (91), while various other studies did not find a significant association between facial

symmetry and attractiveness (45, 56, 67, 92). These inconsistent findings between studies measuring symmetry could partly be attributed to most studies' failure to isolate fluctuating asymmetry, which indicates developmental stability, from directional asymmetry, which does not (87, 93, 94). Simmons *et al.* (89) measured both fluctuating and directional asymmetry and showed that directional asymmetry did not affect attractiveness judgements. Fluctuating asymmetry (and random deviations from directional asymmetry) contributed to the perception of attractiveness in men but not women faces (89). It is interesting that the association between symmetry and attractiveness is more applicable in male, compared to female faces (60, 89). Other facial features (*e.g.* increased facial fat deposits) might influence measures of symmetry. Therefore the findings in less symmetrical individuals may not be due to developmental instability but could be attributed to additional fat deposits in the face. Since women generally have a higher percentage body fat than men (95, 96), this explanation would be consistent with the pattern of results observed by Simmons *et al.* (89) and Komori *et al.* (97) In agreement with this point, Hume and Montgomerie (20) found that Body Mass Index (BMI) negatively predicts facial symmetry in women, but not men.

Another reason studies testing the relationship between symmetry and attractiveness have produced inconsistent results may be due to the method used to evaluate symmetry. Symmetrical faces are consistently judged more attractive when symmetry is estimated by rating unmanipulated faces for symmetry (80, 98-101), or the 'perceptual' technique whereby left-left and right-right chimeras are constructed of the same face and then rated for similarity (53, 54, 69). Similarly, studies that manipulate symmetry by transforming faces along a symmetry continuum normally find that symmetrical faces are judged more attractive (54, 55, 80, 102). However, studies which produce symmetrical images by cutting the face along the facial midline and joining one side of the face and its mirror image together to form chimeras generally find a preference for asymmetry (103, 104). Despite the inconsistencies there is enough evidence to suggest that symmetry is attractive (particularly in men), although symmetry only accounts for ~25% of the variance in attractiveness (39).

1.2.3. Association between Facial Symmetry and Health

Recent studies have implicated perceptions of health in attraction to symmetric faces (79, 105). Jones *et al.* (79) and Rhodes *et al.* (56) show increasing facial symmetry also increases ratings of apparent health, suggesting that symmetry is a cue to health. Researchers have shown that regardless of the methodology used to assess symmetry (measured or rated), symmetrical faces are judged as being healthier than their asymmetrical counterparts (12, 50, 54, 68, 79, 105).

The relationship between facial symmetry and actual health is less clear. Shackelford and Larsen (92) reported a significant association between facial symmetry and some self-reported measures of health (*e.g.* frequency of headaches, trouble concentrating *etc.*), but not others (*e.g.* runny nose, muscle soreness and sore throat) among a cohort of university students. Thornhill and Gangestad (42) found that men with more symmetrical faces reported significantly lower number of respiratory infections but not stomach infections, or antibiotics use. In contrast there were no significant associations between facial symmetry and self-reported health measures in women (42). Similarly, Roberts *et al.* (45) did not find a significant association between measured symmetry and HLA heterozygosity in male faces. The evidence supporting the link between facial symmetry and actual health is therefore fairly limited, particularly in female faces.

1.2.4. Averageness

Facial averageness refers to how similar a face is to the majority of other faces within a population (106, 107). Faces that are non-average have more extreme characteristics than the average of a population. People with more average faces supposedly perform better at tasks such as chewing and breathing (106). Averageness might also denote HLA heterozygosity (14). Similarly to symmetric faces, average faces are thought to reflect good resistance to pathogens and other stressors during development (14, 82, 108).

1.2.5. Associations between Averageness and Attractiveness

A number of studies have found a positive link between facial averageness and attractiveness (29, 50, 54, 56, 58, 61, 64, 97, 99, 109-111). A review by Little *et al*. (90) argues that average faces are considered attractive because average faces are also more symmetrical. Thus several studies were conducted that controlled for facial symmetry. Rhodes *et al.* (100) manipulated symmetry and averageness independently. They showed that both symmetry and averageness positively and independently influence attractiveness judgements (100). In addition various other studies showed that symmetry has a minimal contribution to the attractiveness of average faces (54, 64, 109, 112). Foo *et al*. (50) found a significant positive relationship between averageness and male attractiveness in a Caucasian population. In other words, average faces are independently considered attractive without the influence of facial symmetry.

1.2.6. Associations between Averageness and Health

Average looking faces are generally perceived to be healthier (43, 50, 56, 105, 109). Rhodes *et al.* (56) found that faces which were manipulated to appear more average increased perceived health in both sexes among Western, Asian and Japanese faces. Rhodes *et al.* (105) however found that perceptually more average female, but not male faces were judged as healthier.

Facial averageness is inconsistently associated with actual health (56, 105, 109). Rhodes *et al.* (56) showed that non-average faces were significantly associated with poor childhood health (determined from health scores based on medical records) in men (but not women), poor adolescent health in women (but not men), and did not correlate with mid-adult health scores in either sex. Although a link between facial averageness and health has been found it is far from consistent and more research is required in this area.

1.2.7. Sexual Dimorphism

Human (men and women) faces differ in their shape and appearance (64, 66, 113). The mature facial features distinguishing men and women faces reflect the

masculinization or feminization of secondary sexual characteristics that occurs at puberty (66, 114, 115). Sexually dimorphic traits in human faces might also reflect health during development (42, 66, 105, 116). Masculinization or feminization arises due to the action of hormones such as testosterone in men and oestrogen and progesterone in women (38, 114, 117). Due to the effects of testosterone men are also more susceptible to parasitic infections than women (118-122). Furthermore, the immunocompetence-handicap hypothesis states that the expression of the secondary sexual traits signals health in males because only individuals in good health can withstand the immunosuppressive costs of testosterone (8, 116, 123). This immunocompetence-handicap hypothesis could explain why masculine men are thought to have good genes. Similarly to testosterone, high levels of oestrogen in feminine faces are immunosuppressive (8, 124), thus the ability of the individual to sustain this handicap (8) might be an indication of good genes in feminine faces. In women, femininity may also be linked to fertility through an association with oestrogen (24).

1.2.8. Associations between Sexual Dimorphism and Attractiveness

Feminine female faces are consistently judged more attractive in studies measuring women's facial features from photographs (12, 24, 59, 125) and studies that manipulated facial composites (66). The link between masculinity and attractiveness in male faces is less clear. Several studies found that masculine looking men are considered more attractive (12, 50, 54, 57, 70, 126-129). Other studies have shown that male faces with feminine characteristics and faces of low dominance are considered more attractive (64, 66, 126, 128, 130, 131). Studies by both, Rhodes *et al.* (116) and Rantala *et al.* (129) did not find a significant association between sexual dimorphism (facial masculinity) and attractiveness in adolescent male faces (116) and in the faces of Latvian men (129) respectively. In other words different studies found a preference for masculinity, femininity and no significant preference for sexual dimorphism in male faces. One possible explanation for the differences between studies could be that women's preferences for masculinity change depending on their condition. For example, studies found that women in the follicular phase of their menstrual cycle — when women are more fertile — were significantly more likely to choose a masculine face than those in menses or the luteal phase

(115, 132). Another study has shown that preferences for masculine faces may be determined by experiences and perceptions. Borras-Guevara *et al*. (133) found that women who were exposed to violence (felt more in danger or had experienced more robberries) had lower preference for masculine Salvadoran male faces but not European male faces.

1.2.9. Associations between Sexual Dimorphism and Health

Feminine female faces are generally judged as healthier (24, 116, 134). It is less clear whether feminine facial traits are associated with actual health. Thornhill and Gangestad (42) found that the femininity of young adult faces was weakly related to the number and duration of self-reported respiratory, but not stomach and intestinal infections nor antibiotic use in women. In contrast, a study conducted in women born in the 1920's did not find a significant association between rated femininity of late adolescent female faces and medically assessed adolescent health scores (116). Law-Smith *et al*. (24) found significant positive correlations between late follicular oestrogen and facial femininity and health judgements, while the correlations between luteal progesterone and health judgements were also marginally significant (24). Studies conducted in animals suggest that while oestrogen suppresses cell-mediated immunity it may enhance humoral immunity (124). Excessive levels of oestrogen in older women have been linked to breast, endometrial and ovarian cancers (135-137). In other words, while it may be beneficial to have high oestrogen levels at a young age, the effects are not beneficial as one gets older. Based on the above mentioned studies it may seem as though feminine traits may be poorer signals of actual health.

Masculine male faces are not consistently judged as healthier. Several studies found a positive relationship between rated masculinity and perceived health (105, 116, 134). Scott *et al.* (134) found that rural Malaysians judged masculine faces healthier when faces were manipulated along a sexual dimorphism continuum, whilst Penton-Voak *et al.* (138) did not find a significant association between masculinity and perceived health. Penton-Voak (138) provided very little information about the male images used in the study, making it difficult to compare methodologies across studies and give possible differences for the inconsistencies obtained. Several other

studies found no significant association between masculinity preferences and preferences for perceived health in faces manipulated along an apparent health and masculinity continuum (139-141).

The relationship between masculinity and actual health measurements is fairly consistent. Thornhill and Gangestad (42) showed that highly masculine men reported significantly fewer antibiotic use and lower incidence of respiratory diseases, but not stomach and intestinal infections, than less masculine men. Rhodes *et al.* (116) found that rated masculinity in adolescent male faces correlated significantly with health scores, which was based on detailed medical examinations and health histories. Masculine males (bodies, with similar trends for faces) had more sexual partners, particularly short-term partners, than their less masculine counterparts (99). Given that male reproductive success depends more on short-term mating opportunities than does female reproductive success, these findings suggest that more masculine individuals have higher mating success and increased reproductive potential than their less masculine counterparts (99). Apicella *et al.* (142) found that voice pitch (a sexually dimorphic trait) positively predicted reproductive success. The study found that men with low voice pitch (masculine) have higher reproductive success and more children born to them (142). Rantala *et al.* (129) found a significant association between facial masculinity and hepatitis B antibody response (direct measure of immunity) in Latvian men. This study, however, also found that adiposity and not masculinity mediated the relationship between attractiveness and hepatitis B antibody response in the faces on Latvian men. Lie *et al.* (43) did not find a significant relationship between facial masculinity and MHC (or HLA) diversity, which is an indirect measure of immunity. The relationship between masculinity and more direct measures of immunity is therefore still unclear. A recent study showed that masculinity positively predicted semen quality in Caucasian men (50), this finding suggests that masculinity might be a good signal of male fertility which is an actual measure of health.

1.2.10. Skin Colour /texture

Colouration has been reported as an important component of sexual selection in many species. For example the colour red is associated with dominance in fish

(143), birds (144), and non-human primates (145, 146) and consequently, is linked to attraction to the opposite sex. Research conducted on non-human primates shows that when colour is experimentally manipulated, female rhesus macaques prefer images of redder male faces (146), while males prefer images of redder female hindquarters (147). Studies conducted in mandrills shows that females sexually present more frequently to brighter males and also groom them more frequently (148) suggesting that they are perceived as attractive and healthier.

In humans there are several reasons why skin colour/texture could play a role in attracting the opposite sex. Highly homogenous skin tone could indicate youthfulness (i.e. less UV damage and reproductive hormones (67, 68, 149). Skin lightness indicates youth, parity and hormonal status as the skin darkens with age, during pregnancy and in the infertile phase of the menstrual cycle (150, 151). A redder skin tone has been reported to indicate fertility in non-human primates (152) and increased blood circulation in humans (67, 72). Skin yellowness has also been associated with increased carotenoid (i.e. yellow, orange and red pigments obtained from fruit and vegetables) intake which indicates a healthier diet and appearance (153, 154).

1.2.11. Associations between Skin Colour and Attractiveness

Jones *et al.* (79) found that apparent health of facial skin is positively correlated with ratings of male facial attractiveness. Fink *et al.* (67) found that the homogeneity of skin colour is positively related to attractiveness. The Fink *et al.* (67) experiment was replicated in a wider range of women and in three-dimensional facial images and the homogeneity of skin colour was found to be indeed attractive (68, 149). Similarly, another study by Coetzee *et al.* (155) found that increased skin homogeneity and skin colour independently and significantly contributed to attractiveness judgements of African female faces. Furthermore, the study found that it was the increased preference for a lighter, yellower skin colour that drove this attractiveness preference. HLA- heterozygous men also have healthier appearing skin and are significantly perceived as attractive (45).

Historically pale skinned women were considered more attractive in a wide range of cultures (151), however Smith *et al.* (156) found that in modern Caucasian women's bodies, darker skinned women were considered more attractive. Similarly darker skinned Caucasian faces were judged as slightly more attractive, although the association was not significant (67). Skin darkening in Caucasian women is attributed to exposure to sunlight, when melanocytes increase their production of melanin, darkening the skin (151). In the African/ African American culture light skinned individuals (more yellower) are perceived as attractive and healthy (155, 157). Stephen *et al.* (158) tested the association between skin colour and attractiveness in African and Caucasian men. The study found a significant association between facial attractiveness and skin colour when participants judged faces from their own ethnic group (159). In African raters greater attractiveness was predicted by increased yellowness and increased lightness (158) Skin yellowness has proven to be a rather important and consistent aspect of facial attractiveness in the African population. It is not only related to attractiveness but also to other attractive traits such as sexual dimorphism (160).

1.2.12. Associations between Skin Colour and Health

Several studies show that skin colour plays an important role in health judgements. Matts *et al.* (149) found that the homogenous skin colour distribution of younger looking women was judged as healthier. Furthermore, a somewhat redder skin tone appears healthier (72, 161). Researchers suggest that perceptions of healthy, oxygenated blood may drive associations between skin redness and healthiness (72, 76, 154, 158). A somewhat yellower skin tone has also been associated with healthy appearance (50, 161, 162). Yellowness gives a perception of health due to its association with diet, through carotenoids which are absorbed during intake of fruits and vegetables (153, 154, 163). Roberts *et al.* (45) tested the relationship between skin colour and actual health by testing the relationship between HLA heterozygosity and apparent health judgements of skin patches in male faces, and found that the two are positively related. HLA heterozygosity is positively associated with apparent skin condition, independent of facial shape information (45).

1.2.13. Facial Adiposity

Weight plays an important role in survival and reproduction. Body mass index (BMI; weight scaled for height) and percentage body fat have been reported as important cues to health and attractiveness in human bodies (13, 73, 77, 129, 156, 164-166). The World Health Organization classifies the BMI range as underweight (BMI < 18.5 kg/m^2), normal weight (18.5 – 25 kg/m^2), overweight (25 – 30 kg/m^2) and obese (BMI > 30 kg/m^2; (167), with the normal weight groups generally considered the healthiest (167). Zaadatra *et al.* (168) showed that underweight women are less likely to conceive. Several studies have also shown that women with low fat reserves frequently present ovulatory cycles which hinder reproduction on the most basic level (169-172).

Fat reserves serve as an important determinant of survival, especially during periods of famine (173, 174). Obese individuals can survive famines longer than lean individuals (173). This could be the reason why women — who tend to have higher fat reserves than men (175) — survive famines longer than men (176). Individuals with protein-calorie malnutrition have been shown to be less immunocompetent (177, 178) and more prone to infectious disease (178, 179). Excess fat has also been linked with increased risk of mortality and increased chances of developing cardiovascular diseases and type 2 diabetes (180-182). An intermediate degree of adipose tissue is therefore beneficial. There is substantial evidence linking BMI to health, survival and attractiveness.

1.2.14. Associations between Facial adiposity and Attractiveness

Facial adiposity is significantly associated with attractiveness in Caucasian (20, 73, 77, 129) and African populations (155). In studies conducted on Caucasian groups a curvilinear relationship between facial adiposity and attractiveness is generally found, in that both overweight (BMI >25 kg/m^2) and underweight individuals (BMI <18.5 kg/m^2) from both sexes are judged as less attractive compared to the normal weight (BMI between 18.5 and 25 kg/m^2) counterparts (73, 77, 183). Earlier work in Caucasian populations did not find such consistent results. Hume and Montgomerie (20) found that Caucasian women, but not men, with a higher BMI are judged as facially less attractive. Thornhill and Grammer (184) did not find a significant

association between BMI and female facial attractiveness. More recently, Foo *et al.* (50) found a negative linear relationship between facial adiposity and male and female attractiveness in a Caucasian population, in other words as adiposity increased, individuals were judged as less attractive. The differences between the findings above may firstly be due to the fact that the relationship between facial adiposity and attractiveness is curvilinear a fact that Hume and Montgomerie (20), but not Thornhill and Grammer (184) took into account in their analysis. Secondly, attractiveness judgements might be influenced by media. It has been shown that it is a pervasive and influential communicator of sociocultural standards of attractiveness (185) that portrays a slim body ideal for women.

Different populations might find different levels of adiposity optimally attractive. Tovee *et al.* (186) tested the attractiveness preferences of Africans living in rural South Africa, Africans that were born and raised in Britain and Africans that moved from South Africa to Britain (18 months prior to the study). All three groups differed significantly in the BMI they found most attractive in Caucasian bodies. Rural South Africans prefer the highest BMI (27 kg/m^2), British born Africans prefer the lowest BMI (21 kg/m^2) and the African migrants prefer an intermediate BMI (24 kg/m^2; (186). Coetzee *et al.* (155), however, found a linear relationship between urban black South African women's facial adiposity and attractiveness judgements, in that women with lower facial adiposity were judged as more attractive than their heavier peers. These findings might indicate that optimum adiposity preferences are adjusted facultatively depending on the environment and culture or the media.

1.2.15. Associations between Facial Adiposity and Health

Overweight individuals have been reported to have increased risk of respiratory problems, diabetes mellitus and stroke (181, 187, 188). Obesity has been associated with immune dysfunction as a result of T and B cell impairment (189). Whilst underweight individuals have been shown to seek more health services, have increased mortality and poorer mental health (187) and decreased immunity (178) as compared to their normal-weight counterparts.

Several studies have shown significant linear relationships between facial adiposity and perceived health (73, 74, 77). Coetzee *et al.* (73) found that facial adiposity is significantly associated with cardiovascular health (which was measured using systolic and diastolic blood pressure), self-reported respiratory infections and antibiotics use (73). Tinlin *et al.* (77) found significant correlations between women's facial adiposity and poor psychological health (stress as measured by the stress scale), and low salivary progesterone levels. Some studies have tested the link between facial adiposity and actual health (73, 74, 77, 129). In a large study of 3,130 participants, Reither *et al.* (190) found a significant and positive association between facial adiposity and a variety of poor health indicators, including a significant association between facial adiposity and increased mortality. Moreover, Rantala *et al.* (49) showed that facial adiposity is significantly and negatively associated with antibody response to Hepatitis B vaccination, a direct measure of immunity. Facial adiposity is therefore consistently associated with actual health and explains a substantial amount of the variance of perceived health and attractiveness; thus studies focusing on other facial cues for example symmetry, masculinity would benefit if they controlled for facial adiposity.

1.3. Immunity

The immune system is made up of cells and molecules with specialized roles to defend the body against infection (191). There are two types of responses to pathogens namely: Innate (non-specific cell associated responses) and Adaptive responses (cell-mediated and humoral responses) (191). Innate responses use phagocytic cells (neutrophils, monocytes and macrophages), which release inflammatory mediators and Natural Killer (NK) cells (192). The molecular components of innate responses include complements, acute-phase proteins and cytokines. Phagocytes function in the host in sequential steps: activate recruitment of the cells to the sites of infection, recognition of microbes and ingestion of the microbes by the process of phagocytosis, and the destruction of ingested microbes (193). In addition, phagocytes produce cytokines that serve many important roles in innate and adaptive responses and tissue repair (193). Adaptive responses involve the proliferation of antigen-specific B- and T- cells, which occurs when surface

receptors of these cells bind to antigens. B-cells, activated by T-cells are responsible for making antibodies and can eradicate intracellular pathogens by activating macrophages and killing virally infected cells (192). Here we focus specifically on three aspects of immunity: cytokine expression by peripheral blood mononuclear cells (PBMCs), C-reactive protein and the HLA system.

1.3.1. Peripheral Blood Mononuclear Cells

Studies of the human immune system rely heavily on the assessment of PBMCs. Thus it is important to know what populations are represented in peripheral blood and their distribution (194). PBMCs consist of lymphocytes (T-cells, B-cells, and NK cells), monocytes, and dendritic cells (194). In humans, the distribution of these cell populations differ across individuals (194). Researchers have reported that typically in healthy adults, monocytes can vary from 2 to 10% of PBMCs (195), and within the lymphocyte subset, the relative proportion of T-lymphocytes and B-lymphocytes can range from 61–85% and 7–23%, respectively (196). The lymphocyte population includes 70-85% CD3+ T-cells (45-70% PBMCs), 5-20% B-cells (up to15% of PBMC) and 5-20% NK cells (up to 15% of PBMC) (197). The CD3+ compartment is composed of CD4+ (25-60% PBMC) and CD8+ T-cells (5-30% PBMC) (197). CD4+ T-cells are known as helper T-cells and can be further classified into various subtypes based on the expression profiles of specific cytokines, surface markers, or transcription factors (198). These include regulatory T- cells, T-helper (Th) 1, Th2 and Th17 cells as well as other described subpopulations such as Th9, follicular helper, and TR1 types (198).

The Th1 signature cytokine is interferon-gamma (IFN-γ), while Th2 cells mainly secrete interleukin (IL)-4, IL-5 and IL-13. Th17 cells produce IL- 17A, IL-17F and IL-22 (198). Monocytes are the first cell type to enter peripheral blood after leaving the marrow (193). The cells are incompletely differentiated at this stage and only mature and become macrophages once they enter the tissues (193). These then function as phagocytes as they assume different morphologic forms after activation by external stimuli/ microbes (193). The circulating B-cells include transitional, naïve, and memory subtypes as well as plasmablasts. The role of B-cells is to produce antibodies whose role is to recognize and attach to specific sites on antigens to

block their effect (199). The dendritic cells consist of plasmacytoid dendritic cells as well as myeloid derived dendritic cells. The role of dendritic cells in the periphery is to capture and process antigens, express lymphocyte co-stimulatory molecules, dendritic cells also migrate to lymphoid organs and secrete cytokines to initiate immune responses (200).

1.3.2. Cytokines

Cytokines are regulatory proteins that are produced by a broad range of cells including macrophages, B- and T- lymphocytes, mast cells, endothelial cells, fibroblasts and various stromal cells (Figure 1; 201). They play an important role in the interaction between cells of the adaptive and innate immune system and regulate the body's response to disease and infection (Figure 1; 202). There are a large number of different cytokines. Although they are numerous, cytokines can be functionally divided into two groups: those that are pro-inflammatory and those that are essentially anti-inflammatory (203). The roles of cytokines are classified based on their secretion pattern either by Th1, Th2, Th17 and T-regulatory cells amongst others. Th1 cells secrete IFN-y, IL-2 and lymphotoxin and are known to drive protective immune responses in infectious diseases while Th2 cells produce IL-4, -5, -6, -9, -10 and -13 (204). Here we focus specifically on eight (Table 1) well studied cytokines that represent both pro-inflammatory and anti-inflammatory cytokines: (IL) -10, -6, -2, -8 and -4, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ and tumour necrosis factor-alpha (TNF-α).

Table 1: Summary of immune parameters measured and their various activities

Immune Measure	Activity
INF-y	Critical for innate and adaptive immunity against viral, some bacterial and protozoal infections.
TNF-α	Involved in the activation of the anti-mycobacterial activities of macrophages
IL-2	Plays a central role in the activation and proliferation of lymphocytes
IL-4	Regulates antibody production, haematopoiesis and inflammation
IL-6	Stimulates antibody secretion
IL-10	Suppresses monokines production and reduces cytokine production by Th1 cells
GM-CSF	Stimulate stem cells to produce granulocytes and monocytes
IL-8	Plays a role in inflammation and wound healing
CRP	Used to monitor various inflammatory states
HLA	Serve as antigen presenting molecules.

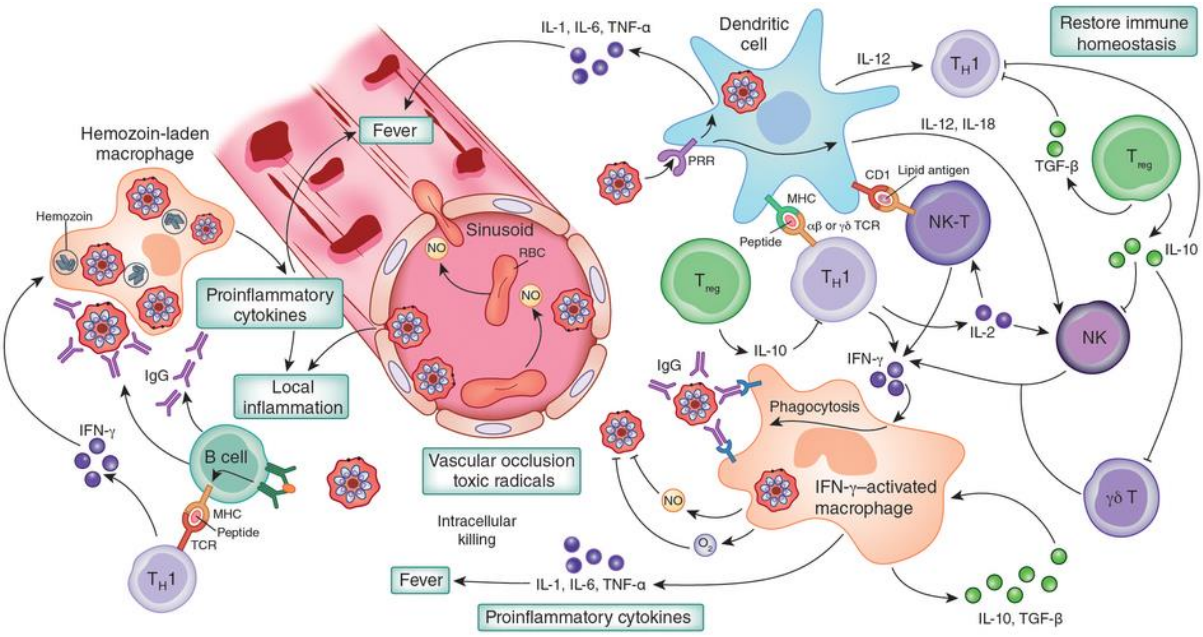

Figure 1: Image depicting Induction of humoral and T cell–mediated immune responses. *The image illustrates the overall involvement of cytokines in the immune system. Image obtained from - Immune mechanisms in malaria: new insights in vaccine development by: Eleanor M Riley and V Ann Stewart, Nature Medicine 19, 168–178 (2013) doi:10.1038/nm.3083*

1.3.3. Pro-inflammatory cytokines

IFN-γ is a pro-inflammatory cytokine that is very important for both innate and adaptive immunity against bacterial and viral infections (205). It is produced by NK and NK T (NKT) cells as part of the innate immune response and by CD4+ Th1 and CD8+ cytotoxic T-lymphocytes (CTL) effector cells once antigen-specific immunity develops. IFN-y is also an activator of macrophages and inducer of class II Major Histocompatibility Complex (205). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor and immune modulator (206). It is produced by T-cells, macrophages, endothelial cells and fibroblasts and many tumour cells upon receiving immune stimuli (207). It plays a pivotal role in various human inflammatory diseases such as rheumatoid arthritis, inflammatory renal disease and inflammatory lung disorders (208). One major function of GM-CSF is to stimulate stem cells to produce granulocytes and monocytes, monocytes migrate to sites of infection and mature into macrophages and dendritic cells. This leads to an increase of macrophages essential for fighting infections (209).

TNF-α is a pro-inflammatory cytokine. It is produced by activated macrophages, CD4+ lymphocytes, NK cells , neutrophils, mast cells, eosinophils, and neurons (210). It is involved in the activation of the anti-mycobacterial activities of macrophages (210) and plays a role in granuloma formation (211). Granuloma formation at the site of mycobacterial infection is an important component of host immunity for controlling infection (212). IL-2 is a Th1 cytokine that plays a central role in the activation and proliferation of lymphocytes that have been primed by antigens. It is pivotal for the expansion of most T-cells, NK cells, and B-cells during various phases of their responses (213). IL-8 is a chemokine that plays a role in inflammation and wound healing (214). It recruits T-cells and other nonspecific inflammatory cells to sites of inflammation and this is done by activating neutrophils (215).

1.3.4. Anti-inflammatory cytokines

IL-10 is an anti-inflammatory cytokine that is produced by alternatively activated macrophages, dendritic cells, Th2 and subsets of T-regulatory cells (216). IL-10 has been reported to regulate the production of IL-12 and decrease IFN-γ production and regulates antigen presentation (217). IL-10 also activates B-cells and Th2 type cells while inhibiting Th1 type cytokine production (218, 219). IL-4 is a prototypic cytokine which has an important role in regulating antibody production, haematopoiesis and inflammation, and the development of effector T-cell responses (220). IL-6 has long been regarded as a pro-inflammatory cytokine, but has both pro- and anti-inflammatory properties. IL-6 has a diverse effect on the regulation of immune responses, inflammation, oncogenesis, and haematopoiesis amongst others and is widely known as an inducer of the acute phase response (221). Cytokines play an important role in the immune response and will be investigated in our study as a direct measure of immunocompetence.

1.3.5. C - reactive protein (CRP)

C-reactive protein, is a hepatic acute phase protein which is regulated by circulating levels of interleukin-6 and predicts coronary heart disease incidence in healthy subjects (222). CRP levels rise in response to inflammation and physiologically CRP

binds to phosphocholine which is expressed on the surface of dying cells and other bacteria to activate the complement system via the CQ3 complex to enhance phagocytosis by macrophages (223). It is also believed to play an important role in innate immunity as an early defense system against infections (224). CRP levels have been reported to rise drastically during the vast inflammatory processes in the body (224).

1.3.6. Human Leukocyte Antigen Complex

The MHC was discovered for its role in transplant rejection (225, 226). In humans this complex plays an essential role in both the humoral and cell-mediated immune response where they serve as antigen presenting molecules (227). HLA loci are located on chromosome 6 (228) and consists of three classes *viz*. I, II and III (229). HLA class I protein is composed of two chains: α chain and β2 microglobulin (230). The α chain consists of a transmembrane region and three extracellular domains: α1, α2, and α3. The HLA class I molecule is expressed on the membrane of all nucleated cells (230). The class I HLA genes are found on lymphocytes and myeloid cells which are common on liver, lung and kidney cells (231, 232). These class I HLA genes code for glycoproteins which play a crucial role in the immune response presenting self and non-self peptides to CD8+ T-cells (233). CD8+ cells are then activated to produce clones that destroy similarly infected cells (227). Class I genes are divided into classical (HLA-A, B and C) and non-classical (HLA-E, F, G, H, J, X, MICA-MICE) genes.

HLA class II molecules have an immunoglobulin-like structure. It consists of one α chain and one β chain (230). Each chain contains a transmembrane region and two extracellular domains (α1 and α2 in the α chain, β1 and β2 in the β chain) (230). HLA class II proteins are expressed on the membrane of antigen presenting cells and are responsible for presenting extracellular antigens (230). Class II HLA genes are also divided into classical (HLA-DR, DP and DQ) and non-classical (HLA-DM, DN and DO) genes. They code for glycoproteins that are located on macrophages, dendritic cells and B cells (234). Class II HLA molecules present processed peptides to CD4+ helper T-cells. The binding of CD4+ cells to HLA complex activates macrophages triggering antibody secretion by B-cells (227). Class III HLA genes play an important

role in the immune system as they code for several genes which are involved in the activation cascades of the complement system (C2, factor B, C4) (229).

1.3.7. HLA Diversity

In order for the immune system to successfully ward of disease it needs to have the ability to recognise different types of pathogens. HLA molecules are highly diverse, which allows them to recognise a large number of varying pathogens (235). There are several ways in which this diversity can be accomplished. First, the HLA molecule is extremely polymorphic, there are multiple variants of each gene within the population as a whole (235). In 2005 the IMGT/HLA sequence database reported 396 HLA-A and 699 HLA-B alleles (236). The numbers have since increased to 3399 HLA-A and 4242 HLA-B alleles (237). Second, HLA molecules are polygenic: they contain several different HLA class I and HLA class II genes, so that every individual possesses a set of HLA molecules with different ranges of peptide-binding specificities (235). Third, Individual HLA molecules can bind multiple peptides and the same peptide can be bound by more than one HLA molecule (238). Fourth, the diversity of HLA molecules is further increased through co-dominant expression. For example, in heterozygous individuals both parental alleles are expressed on all cells (232). Fifth, HLA molecules are represented on almost every cell in an organism, this will enable the recognition of increased pathogens regardless of the affected cell (239). Sixth, Janeway and Travers (235) reported that the diversity of HLA class II molecules is greatly increased by the association of multiple β with α chains. Thus class II rearrangements play a role in increasing HLA diversity.

1.3.8. HLA based mating preferences

One of the best studied genetic based mating systems is the HLA/MHC (227). HLA based mating preferences are driven by pathogen driven selection, which is made up of heterozygote advantage, frequency dependent selection and inbreeding avoidance (240). Heterozygote advantage contributes to mating preferences in two forms: Firstly, heterozygote advantage favours HLA disassortative (dissimilarity) mating preferences (i.e. the preference of someone with different HLA alleles to one's own). HLA assortative mating preferences are preferences of someone with

the same HLA alleles. Both HLA disassortative and assortative mating preferences have been investigated. Studies in mice suggest a preference for the scent of HLA dissimilarity (227, 241). In humans HLA disassortative preferences have been investigated using both scent and faces. Wedekind *et al.* (242) found that normally ovulating Swiss woman (not taking a contraceptive pills) preferred the scent of Swiss men who had HLA genotypes dissimilar to their own (242). A second study conducted in a Bernish (Swiss-German dialect) population, replicated the HLA-dissimilarity scent preference study conducted in the Swiss population (242) and found that Bernish men preferred the scent of Bernish women who had HLA genotypes dissimilar to their own (243). Thornhill *et al.* (47) found that men from mixed ethnicities preferred the scent of individuals which were HLA-dissimilar to them. Similar findings were not observed in women from mixed ethnicities (47).

Roberts *et al.* (244) found that British male faces received higher attractiveness scores when judged by British women who were HLA-similar and not HLA-dissimilar to them. Ober *et al.* (245) found that Hutterite married couples tend to be more HLA dissimilar , however studies in South American Indians (246) and a study of Japanese couples (247) reported no evidence of HLA-dissimilar assortative pairing among married couples (for review; 241). This disagreement in the findings may be because HLA genetic variation is structured by ethnicity (248, 249), where population frequencies of HLA alleles depend on geographical location and on the level of population heterogeneity. Other factors such as culture and environmental surroundings might override HLA based preferences in some cultures but not others. The discrepancies could also be attributed to differences in preferences for faces (244) and scents (227).

Secondly, heterozygote advantage favours the preferences for HLA heterozygosity (i.e. the preference for someone who are more HLA heterozygous). The heterozygote advantage hypothesis states that heterozygotes have a higher fitness than their homozygote counterparts as they have the ability to present an increased number of antigen peptides to the immune system (250). Heterozygote advantage is supported by the association of HLA heterozygosity and disease resistance. Chinook salmon that were heterozygous for class II MHC loci had a higher survival rate than homozygotes when exposed to infectious haematopoietic necrosis virus (251). A

study by Thursz *et al*. (252) found that an increase in class II HLA heterozygosity correlated with less persistent infections of hepatitis B in humans (252). Heterozygote advantage will be discussed further in chapter 3.

Frequency dependent selection is made up of two forms. Firstly, it also favours disassortative mating preferences. The moving target hypothesis proposes that HLA disassortative mating preferences provide a moving target to pathogens that successfully evade immune recognition (227). In other words as the pathogen adapts to the parental HLA type, disassortative mating will produce offspring which are different to the parental type and thus increases the chances of survival for the offspring (227). The second form of frequency dependent selection has more to do with the benefit associated with rare / common alleles (240). Frequency dependent selection will be discussed further in chapter 3.

1.4. Consolidating the gaps in the current literature

There are a few gaps in the literature that this study aims to address. First, most studies focusing on the association between facial attractiveness and health have concentrated on indirect measures of health, such as self-reported health measures (20, 41, 42, 92). In my view, self-reported measures may also be affected by gender. For instance, women might be more aware of their health and thus report health scores accurately. Rhodes and Simmons (105) criticized the use of self-reported health measures, stating that they are more prone to be influenced by a person's affective state (less attractive individuals are more likely to recall negative experiences and thus report worse health). It is thus important not to rely solely on self-reported health measures, but to include more direct measures of immunity. We will address this gap by focusing on more direct measures of immunity, such as cytokine profiling, CRP and HLA typing. Second, Rantala *et al.* (49, 129) did test immunity directly, but antibody response is only a small part of the adaptive immune response and provides a small glimpse into overall immunity. The immune measures included in this study, C-reactive protein, cytokine response and HLA typing provides a more holistic view of overall immunity. Third, previous studies investigating the relationship between attractiveness and immunity focused almost exclusively on European and American populations. We will therefore focus on an African

population in this study, more specifically on African males, since the strongest theoretical support is for the association between male facial attractiveness and a healthy immune response (8).

Chapter 2

Facial appearance reveals immunity in African men

2. Introduction

In the previous chapter I provided several lines of evidence that support the notion that facial appearance (health and attractiveness), facial cues *(e.g.* masculinity; symmetry; averageness; facial adiposity and skin colour) are inconsistently associated with both direct and indirect measures of immunity. For example, studies have investigated the link between facial attractiveness and measures of immune response (43, 45, 48, 129). Roberts *et al*. (45) and Lie *et al.* (43) found that heterozygosity at the HLA positively predict male facial attractiveness in young British and Australian men. In contrast, studies in African (46) and Australian (43) women did not find a significant association between HLA heterozygosity and facial attractiveness. More recent studies found a significant positive relationship between a direct measure of immunity (antibody response after Hepatitis B vaccination) and facial attractiveness in European men (129), but not women (49).

Various facial cues might influence the relationship between facial attractiveness and immunity. Roberts *et al*. (45) found significant positive associations between skin condition, specifically perceived skin health, HLA heterozygosity and facial attractiveness in British men. They did not test which aspect of skin condition might be driving this association, but skin yellowness is a likely candidate. Carotenoids — the yellow, red pigments obtained from fruit and vegetables and deposited in the skin — have previously been shown to increase skin yellowness in African and Caucasian skin (153, 253). Furthermore, this increase in skin yellowness is considered healthy and attractive in both populations (153, 155, 158). Carotenoids serve as antioxidants in the body and their levels subsequently reduce after infection (254, 255), indicating a positive link between carotenoid levels and immunity. Lie *et al.* (43) found that averageness, but not symmetry or masculinity, mediate the relationship between HLA heterozygosity and facial attractiveness in Australian men, but not women. Roberts *et al.* (45) also did not find a significant association between

measured facial symmetry and HLA heterozygosity. The immunocompetence handicap hypothesis (ICHH) proposes that androgen-mediated traits accurately signal condition due to the immunosuppressive effects of androgens, such as testosterone (8). Facial masculinity is generally assumed to serve as such a trait, in that masculine men are predicted to have better immune responses, because only men with strong immune systems are expected to withstand the immunosuppressant effects of the high levels of circulating testosterone necessary to develop masculine features (256). A recent review, however, concluded that there is little direct evidence of a link between facial masculinity and immunocompetence in humans (256). Moreover, Rantala *et al.* (129) found that facial adiposity, but not masculinity, significantly mediates the relationship between immunity and attractiveness in Latvian men.

These studies provide valuable insights into the relationship between facial appearance and immunity, but several gaps still remain. First, the immune system is a complex system, consisting of various different subsystems such as the humoral (or antibody-mediated) response and the cell-mediated response (which mostly involves T-cells and responds to any cell that displays aberrant HLA markers; 257). Antibody-mediated response and HLA heterozygosity (tested in previous studies) represent only a small component of the overall immune response. Cytokines are regulatory proteins that are produced by a wide range of immune cells including macrophages, B and T lymphocytes and mast cells (201, 258). They play an important role in the interaction between cells of the humoral and cell-mediated immune responses and regulate the body's response to disease and infection (202). Eight well studied cytokines were selected for this study, representing the Th1 pathway (“cellular immunity”; *e.g.* interferon gamma [INF-y], interleukin 2 [IL-2] and Tumour necrosis factor alpha [TNF-α]; (259), the Th2 pathway (“humoral immunity”; *e.g*. IL-4,-6 and -10; (260), both pathways (*e.g.* Granulocyte-macrophage colony-stimulating factor [GM-CSF]; review; 261) and a chemokine *e.g.* IL-8 (262). Functional cytokine analysis of Peripheral Blood Mononuclear Cells (PBMCs) after Lipopolysaccharide (LPS) stimulation provides a direct measure of immunocompetence (263). C-reactive protein (CRP) is an acute phase reactant, commonly used to evaluate infection, tissue injury and inflammation (264).

Functional cytokine analysis and CRP therefore provide a more comprehensive view of immunity. Secondly, previous studies on the topic, focused almost exclusively on white European or Australian populations (67, 73, 129, 265). To our knowledge, no study has yet tested the association between facial attractiveness and a direct measure of immunity in African men. Thirdly, most previous studies, including some of our own, focused on single facial cues when testing the relationship between facial appearance, health and attractiveness (20, 24, 38), which disregards the interrelationship between facial cues.

The aim of this study was to test the relationship between two direct measures of immunity (functional cytokine profile and CRP), overall facial appearance (attractiveness and health) and the five main facial cues (adiposity, masculinity, averageness, symmetry, skin colour) in African men.

2.1. Materials and methods

2.1.1. Ethics statement

This study was approved by the ethics committee at the University of Pretoria (EC141002-083).

2.1.2. Participants

Ninety two African men (mean age=20.4, SD=3.0; BMI=21.7, SD=3.2) were recruited from the University of Pretoria, South Africa. Each participant provided written informed consent and completed a short questionnaire, including questions on age and ethnicity. Full colour frontal and profile facial photographs were taken with a Canon Eos 40D digital camera under standardized conditions. Participants were asked to maintain a neutral expression. The facial photographs were standardised for orientation and size using Psychomorph and other in-house software. Skin colour measurements were obtained from a subset of individuals (49 participants) on four separate points (right cheek, left cheek, forehead and palm of the hand) using a Konica Minolta CM-2300d Spectrophotometer. The predefined skin areas were measured in CIELab colour space: CIELab L* (luminance axis), CIELab a* (green-

red axis), and CIELab b* (blue-yellow axis) and spectral reflectance values (360 to 740 nm). Higher values on the three axes indicate lighter, redder and yellower colours respectively. The measurement aperture was held lightly against the skin to minimize pressure-induced bleaching. All participants were asked to clean these skin areas with hypoallergenic wipes at least 20 minutes before spectrophotometry measurements. Each colour measurement was taken twice and averaged. Colour values for the forehead and cheeks were averaged to provide facial colour values for further analysis. Participants, height and weight were measured and their BMI calculated (weight/height^2).

2.1.3. Image Ratings

Twenty African females (mean age=22.5, SD=2.2) were recruited from the University of Pretoria Hatfield campus to rate the male facial photographs for attractiveness, health, symmetry, masculinity, distinctiveness and facial adiposity. Distinctiveness ratings were reverse coded to reflect averageness. Facial adiposity was rated and not actually measured (e.g. through skinfold thickness) thus it is 'apparent adiposity'. Each female participant provided informed consent and completed a short questionnaire with basic demographic *(e.g.* age, ethnicity) information. The male facial images were presented in a randomised order on a computer screen and female participants were asked to rate each image on separate 7-point Likert scales (1=very unattractive, 7=very attractive *etc*.). All Cronbach alpha values were > 0.72 (Table 2.1), which indicates high inter-rater consistency and reliability.

Table 2.1: Cronbach alpha values for rated facial cues.

Facial cue	**Cronbach's alpha**
Attractiveness	0.94
Health	0.91
Adiposity	0.94
Masculinity	0.82
Symmetry	0.76
Averageness	0.73

2.1.4. Immunological analysis

Twenty millilitres of blood was drawn by a qualified phlebotomist from a subset of 68 participants, which included the perceived 20 most attractive and 21 least attractive men that agreed to have their blood drawn. These 41 samples were used for cytokine analysis in order to maximise power and reduce cost. Blood samples were collected in 4 ml heparin BD vacutainer® tubes and processed within 2 hours. PBMCs were isolated at the plasma-Ficoll interphase on Histopaque 1077 Sigma-Aldrich) using standard barrier density gradient centrifugation (266). Cells were enumerated using Reichert-Jung Microstar light microscopy (267). Viable cells were stimulated with 0.5 µg/ml of lipopolysaccharide (LPS) and incubated at 37°C in a CO2 incubator (5%) for 16 hours. LPS is a major component of the outer membrane of Gram-negative bacteria (268), it stimulates host cells and makes them produce various pro-inflammatory cytokines eliciting strong immune responses (269). Supernatants were harvested after 16 hours and stored at -20°C until further use.

The levels of eight cytokines: IL-10, -6, -2, -8 and -4, GM-CSF, IFN-γ and TNF-α were evaluated in PBMCs using Bio-Plex Pro™ Assay kits (Bio-Rad Laboratories, Hercules, CA, USA) on the Bio-Plex Suspension Array System (Bio-Rad Laboratories). All samples were analysed undiluted. In short, 50µl of pre-mixed antibody covered magnetic beads were added to each of the wells of a 96 well plate. Standards which were provided with the kits were added to the appropriate wells to facilitate quantification of the selected cytokines. An additional internal control from a volunteer donor was added onto each of the plates for internal quality control. The samples were then added to the designated wells in duplicate. The plates were sealed and incubated with agitation on an orbital plate shaker for 1 hour at room temperature. The fluid was removed and the plates were washed twice using an automated plate washer (Bio-Rad Laboratories).

Detection antibodies were then added to each well of the plate and incubated with agitation on an orbital plate shaker for 30 minutes at room temperature, after incubation and further washing, Streptavidin-Phycoerythrin was added to each well and incubated for a further 30 minutes at room temperature on an orbital shaker. The plates were washed again and the beads resuspended in assay buffer. Prior to being

assayed on a Bio-Plex Suspension Array platform (Bio-Rad Laboratories). Bio-Plex Manager Software 6.0 was used for bead acquisition and analysis of median fluorescence intensity. Results are reported as concentration (pg/ml). CRP levels were determined in the serum of a subset of individuals' using CardioPhase® hsCRP (Siemens) reagents on a BN Prospec Nephelometer (Siemens) as described in Richard and Fogoros (270). Results are reported as mg/ml. Most CRP values were below 1.0 µg/ml, while only 15 participants had CRP values between 1.0µg/ml and 7.3 µg/ml.

2.1.5. Statistical analysis

All analyses were performed in SPSS version 24. Prior to analysis, all variables were examined for accuracy of data entry, missing values, outliers, normality of their distributions and pairwise linearity (271). All values were normally distributed (two-tailed critical z score = ± 3.29, p = 0.001, except facial attractiveness (skewness z=4.46) and CRP (skewness z=11.15, kurtosis z=23.05). Log transformation successfully normalised both distributions (log attractiveness skewness z= 2.07, kurtosis z= -0.57; log CRP skewness z= 2.84, kurtosis z= -0.35). Pearson's correlations (2-tailed) and Principal Component Analyses (PCA) were conducted.

2.2. Results

According to World Health Organisation standards, 14% of participants were underweight, 69% normal weight, 14% overweight and 3% obese (167). Facial adiposity was significantly correlated with BMI ($r=0.628$, $p<0.0005$; indicating that observers were rating facial adiposity appropriately) and showed a curvilinear relationship with attractiveness ($F=4.128$, $p=0.019$, $R^2=0.085$) and health ($F=7.137$, $p=0.001$, $R^2=0.138$). The optimum relative BMI value was 22.4kg/m^2 for both attractiveness and health (as calculated from facial adiposity (183); with under-and-overweight men judged less attractive and healthy). Some of the facial cues were significantly correlated (*e.g.* adiposity and masculinity; Table 2.2). Closer inspection revealed non-linear relationships between adiposity and masculinity. The curvilinear relationship between masculinity (x-axis) and adiposity (y-axis) explained more variance ($F=24.038$, $p<0.0005$, $R^2=0.351$) than the curvilinear relationship between

adiposity (x-axis) and masculinity (y-axis) ($F=18.934$, $p<0.0005$, $R^2=0.301$), although both were highly significant. More masculine faces were therefore also considered heavier, but only up to a point, where after further increases in masculinity no longer made the faces appear heavier (Figure 2.1, 2.2). Squared terms for facial adiposity and masculinity were included in subsequent analysis due to these non-linear relationships.

Table 2.2: Pearson's correlations between attractiveness and health, facial cues, skin colour, cytokine component and CRP.

	Health	Symmetry	Masculinity	Adiposity	Averageness	CIELab L*	CIELab a*	CIELab b*	Cytokine component	log CRP	BMI	Age
Log Attractiveness	.844***	.565***	.407***	.090	.400***	.691***	.631***	.699***	.291$^{\Delta}$	-.085	.056	-.147
	(92)	(92)	(92)	(92)	(92)	(49)	(49)	(49)	(41)	(68)	(92)	(92)
Health	1	.590***	.519***	.147	.406***	.493***	.395***	.490***	.303$^{\Delta}$	-.026	.091	-.086
		(92)	(92)	(92)	(92)	(49)	(49)	(49)	(41)	(68)	(92)	(92)
Symmetry		1	.463***	-.034	.240*	.234	.104	.171	.208	-.185	-.060	-.067
			(92)	(92)	(92)	(49)	(49)	(49)	(41)	(68)	92	92
Masculinity			1	.455***	.075	.146	.108	.159	.274	-.143	.274**	.200
				(92)	(92)	(49)	(49)	(49)	(41)	(68)	92	92
Adiposity				1	-.001	.053	.003	.063	.282	-.007	.628**	.162
					(92)	(49)	(49)	(49)	(41)	(68)	92	92
Averageness					1	.203	.281$^{\Delta}$	.274$^{\Delta}$	-.202	-.002	.014	-.035
						(49)	(49)	(49)	(41)	(68)	92	92
CIELab L*						1	.849***	.961***	.435*	-.203	-.010	.127
							(49)	(49)	(25)	(43)	(51)	(51)
CIELab a*							1	.941***	.363$^{\Delta}$	-.108	-.030	.050
								(51)	(25)	(43)	(50)	(50)
CIELab b*								1	.422*	-.185	-.008	.129
									(25)	(43)	(51)	(51)
Cytokine component									1	-.188	.064	.343*
										(41)	(41)	(41)

log CRP										1	.188	-.070
											(68)	(68)
BMI											1	-.003
												(95)
Age												1

$^{\Delta}p<0.1$, *p<0.05, ** p<0.01, $^{***}p<0.001$. N in Brackets

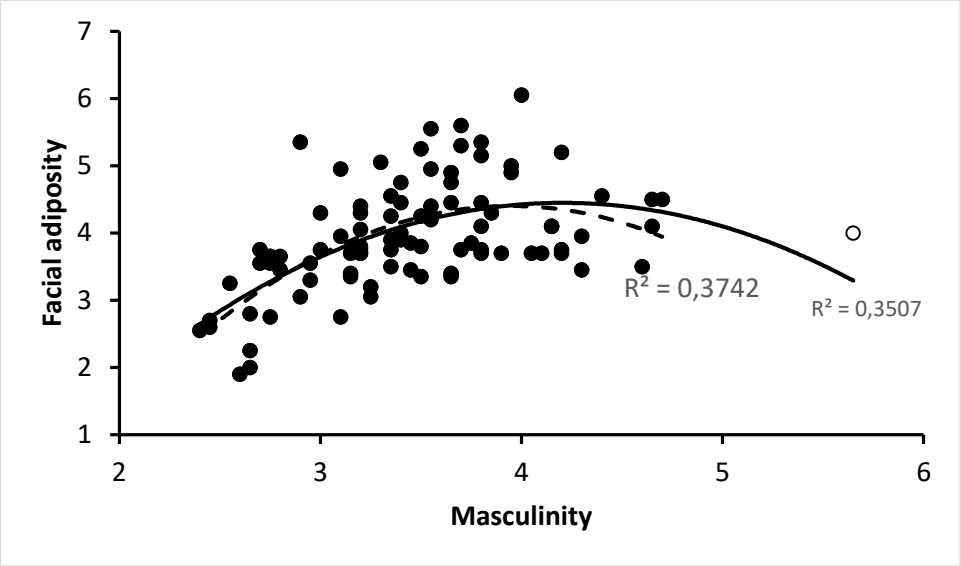

Figure 2.1: Curvilinear relationship between masculinity and facial adiposity. *The curvilinear relationship between masculinity (x-axis) and adiposity (y-axis) explained more variance ($F=24.038$, $p<0.0005$, $R^2=0.351$; solid line) than the linear relationship between masculinity (x-axis) and adiposity (y-axis) ($F=23.457$, $p<0.0005$, $R^2=0.207$) and the curvilinear relationship between adiposity (x-axis) and masculinity (y-axis) ($F=18.934$, $p<0.0005$, $R^2=0.301$). The removal of one potential outlier (white dot with black outline) only strengthened the relationship ($F=26.308$, $p<0.0005$, $R^2=0.374$; dotted line).*

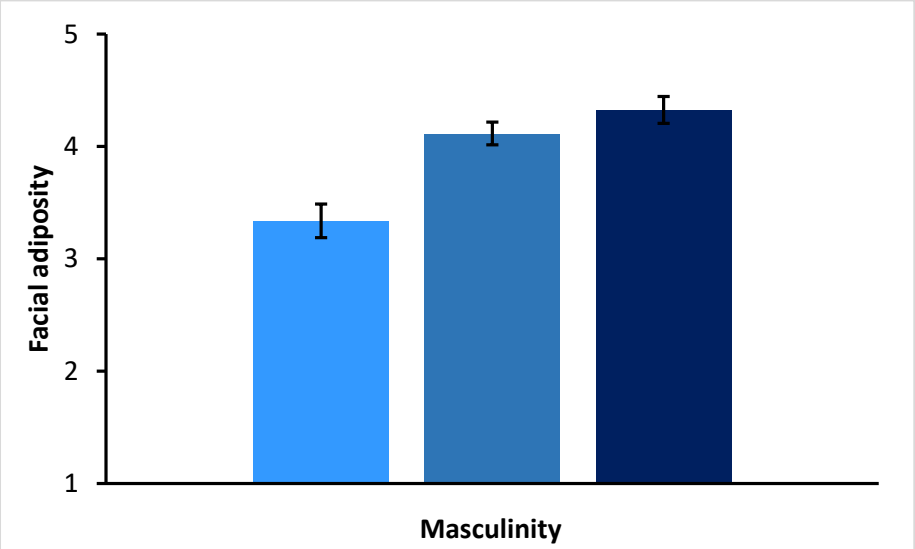

Figure 2.2. Facial adiposity levels for different masculinity groups. *Facial adiposity levels differed significantly between the low and medium masculinity group, but not between the medium and high masculinity group. *** $p<0.0005$. Error bars indicate standard error of the mean.*

Due to the significant correlations between facial cues, a PCA was conducted on symmetry, averageness, masculinity, masculinity^2, adiposity and adiposity^2. Averageness had low communality (0.17) with the rest of the variables and was excluded from the PCA. The PCA produced two principal components with eigenvalue >1, which explained a cumulative variance of 89%. Masculinity (0.90), masculinity^2 (0.87), adiposity (0.78) and adiposity^2 (0.75) loaded highly on PC1 explaining 58% of the variance. PC1 is hereafter known as the masculinity, adiposity component, with higher values indicating more masculine, heavier faces (Figure 2.3, 2.4). Symmetry (0.73), adiposity (-0.60) and adiposity^2 (-0.63) loaded highly on PC2 explaining 30% of the variance. PC2 is hereafter known as the symmetry, low adiposity component, with higher values indicating more symmetrical, skinnier faces (Figure 2.3, 2.5). The masculinity, adiposity component was more highly correlated with facial adiposity measures below ($r=0.742$, $p<0.0005$) than above the median facial adiposity ($r= -0.141$, $p>0.1$), while the symmetry, low adiposity component was more highly correlated with facial adiposity measures above ($r= -0.669$, $p<0.0005$) than below the median facial adiposity ($r=0.464$, $p=0.001$). The masculinity, adiposity component is, therefore, more relevant to underweight and lower normal weight men, while the symmetry, low adiposity component is more relevant to upper normal and overweight men.

The average facial colour values (lightness, yellowness and redness) were also highly correlated (Table 2.2). A PCA of the facial skin colour values produced one principal component with eigenvalue >1, explaining 95% of the variance. Yellowness (0.99), lightness (0.96) and redness (0.96) all loaded highly and positively on PC3, hereafter the colour component. Higher values for this component indicate yellower, lighter and redder skin tone (Figure 2.3). There was no significant association between PC3 and PC1 or PC2 ($p>0.1$).

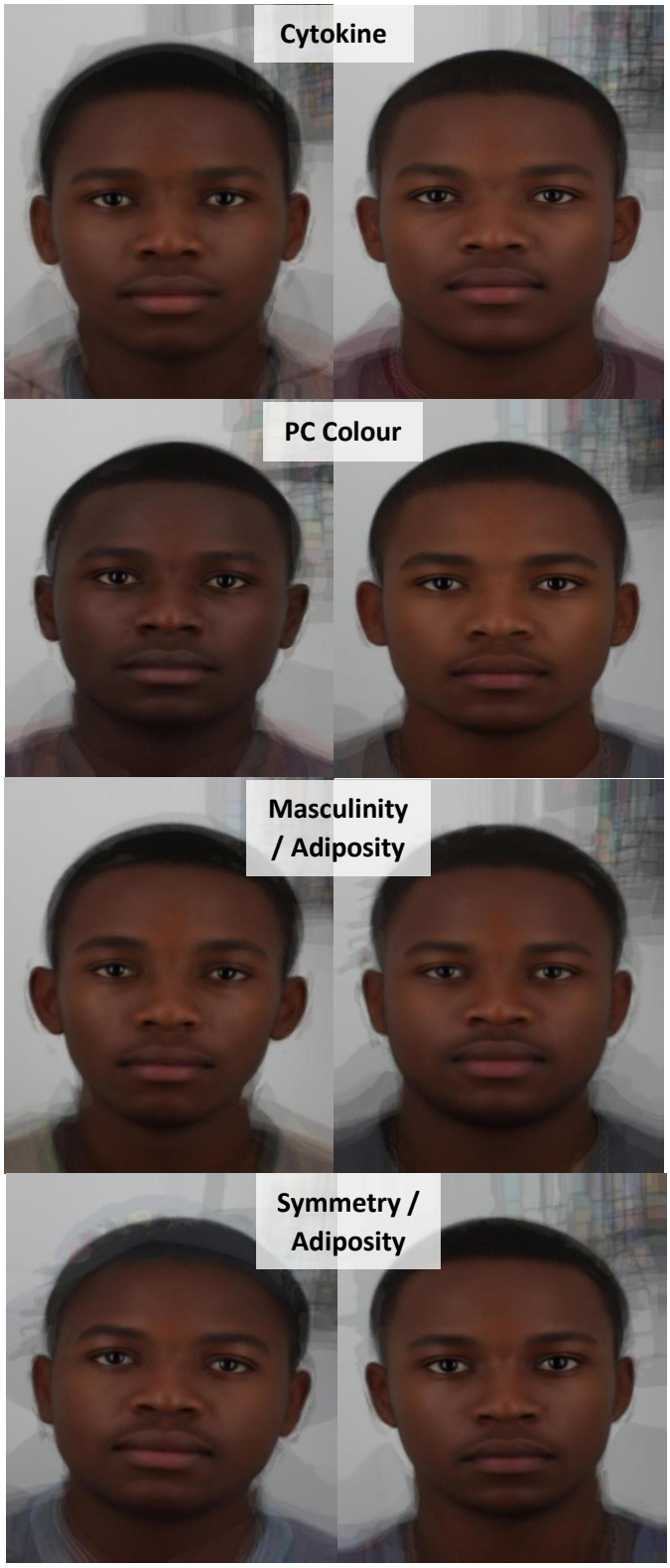

Figure 2.3: Composite images of cytokine response; PC Colour; the masculinity, adiposity component (PC1); and the symmetry, low adiposity component (PC2). *Images on the left are composite images of the ten men with the lowest values for that variable, while images on the right are composite images of the ten men with the highest values.*

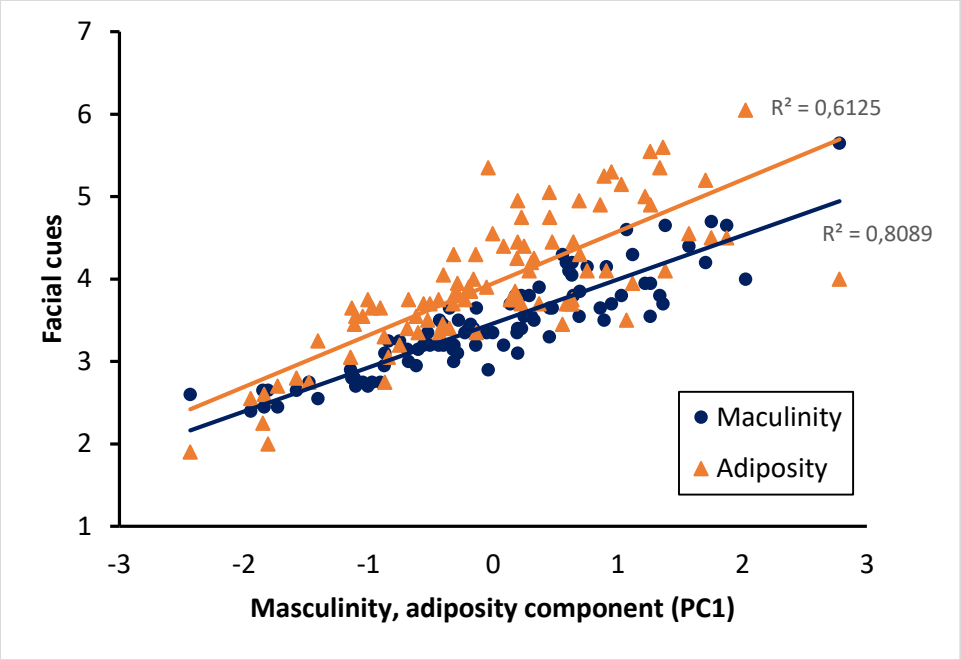

Figure 2.4. The relationship between the masculinity, adiposity component (PC1), adiposity and masculinity.

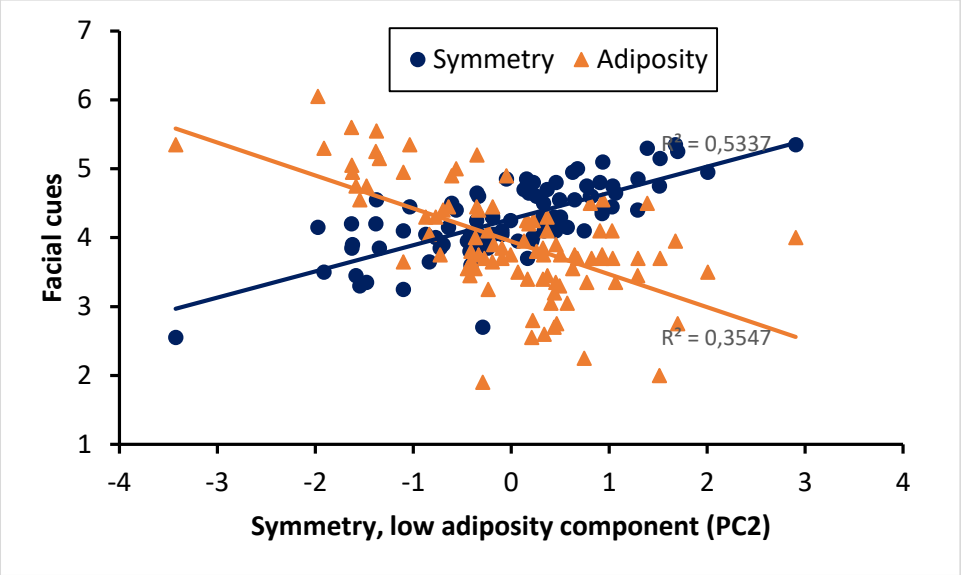

Figure 2.5. The relationship between the symmetry, low adiposity component (PC2) and the facial cues symmetry and adiposity.

As would be expected cytokine levels were generally increased after LPS stimulation (Table 2.3). The individual unstimulated and LPS stimulated cytokine levels were highly positively correlated with themselves and with each other (Table 2.4), indicating that all cytokine levels showed a similar picture and that individuals with

high unstimulated cytokine values generally also had high values after LPS stimulation. A PCA of all the unstimulated and LPS stimulated cytokine levels produced two principal components with eigenvalue >1, which explained a cumulative variance of 85%. All unstimulated and LPS stimulated cytokine values loaded highly and positively on component 1 (>0.73), hereafter known as the cytokine component. Higher values for this component indicate higher unstimulated and LPS stimulated cytokine levels. None of the cytokine values loaded highly on component 2 (<0.51 and >-0.45) with higher loadings for all cytokines on component 1. Component 2 was therefore excluded from further analysis.

Table 2.3: Average cytokine levels before and after LPS stimulation.

Cytokine	**Unstimulated (basal) cytokine level**	**Stimulated (LPS) cytokine level**	**Stimulated - Unstimulated**
IL-2	112.35	241.73	129.38
IL-4	88.52	157.17	68.65
IL-6	6 107.57	11 539.11	5 431.54
IL-8	15 160.09	18 976.78	3 816.69
IL-10	864.33	1 725.50	861.17
GM-CSF	383.29	696.83	313.54
IFN-y	1 077.80	1 997.28	919.48
TNF-α	2 056.37	4 705.26	2 648.89

Table 2.4: Correlations between individual unstimulated cytokine levels *(above diagonal), individual LPS stimulated cytokine levels (below diagonal) and between unstimulated and LPS stimulated cytokine levels (on the diagonal in bold).*

	IL2	IL4	IL6	IL8	IL10	GM-CSF	IFN-y	TNF-α
IL2	.596***	.955***	.985***	.816***	.899***	.885***	.937***	.968***
IL4	.750***	.654***	.949***	.841***	.912***	.913***	.952***	.925***
IL6	.910***	.779***	.552***	.860***	.925***	.870***	.943***	.933***
IL8	.629***	.685***	.660***	.763***	.851***	.752***	.828***	.749***
IL10	.656***	.829***	.708***	.642***	.660***	.886***	.902***	.836***
GM-CSF	.772***	.887***	.825***	.674***	.772***	.566***	.895***	.879***
IFN-y	.776***	.916***	.800***	.633***	.811***	.800***	.676***	.915***
TNF-α	.909***	.736***	.806***	.559***	.583***	.727***	.724***	.585***

***p<0.001. N =41. Interleukin 2 (IL2), 4 (IL4), 6 (IL6), 8 (IL8), 10 (IL10); Granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon gamma [INF-y]; and Tumor necrosis factor alpha [TNF-α].

Age was significantly correlated with the cytokine component (r=0.343, p=0.028) and marginally associated with the masculinity, adiposity component (r=0.197, p=0.059), but none of the other variables (p>0.1). Age was therefore, controlled (using partial correlations in SPSS) for in all subsequent correlations involving these two components. The cytokine component was significantly associated with perceived attractiveness and health (Figure 2.6), indicating that women consider men with a stronger cytokine response more attractive and healthy. In order to identify which structural or colour components mediate these relationships, separate Pearson's correlations for all facial and colour components were performed.

The colour component was significantly associated with perceived facial attractiveness and health and marginally associated with the cytokine component (Figure 2.6). CIELab b* and CIELab L* were more strongly correlated with the cytokine component, attractiveness and health than CIELab a* (Table 2.2). Of the

three colour variables, skin yellowness and lightness therefore showed the most consistent relationship with both immunocompetence and overall appearance. The correlations between the cytokine component and the spectral values in the palm of the hand closely followed the predicted spectral pattern for carotenoids, especially the carotenoid lycopene, but not the predicted spectral pattern for other human pigments (Figure 2.7). A similar pattern was not observed in the face, where the correlations between the cytokine component and the spectral values followed the predicted spectral pattern for melanin, indicating the masking effect of melanin in highly melanised skin (Figure 2.8). The masking effect of melanin on the carotenoid-immune response relationship is further evidenced by a significant association between CIELab b* and the cytokine component above the median for CIELab L* (lighter faces; $r=0.693$, $p=0.026$), but not below the median for CIELab b* (darker faces; $r=-0.245$, $p>0.1$).

The masculinity, adiposity component (PC1) was significantly associated with the cytokine component, facial attractiveness and health (Figure 2.6). In fact, this component was more strongly associated with the cytokine component than BMI was ($r=0.097$, $p>0.1$). The symmetry, low adiposity component (PC2) was significantly associated with facial attractiveness and health, but not with the cytokine component (Figure 2.6), even after the removal of one influential outlier ($r=0.189$, $p>0.1$) Facial averageness was also significantly associated with attractiveness and health, but not the cytokine component (Figure 2.6).

Attractiveness and health were highly correlated (Table 2.2), so similar results were observed for both (Figure 2.6), except that the colour component showed a significantly stronger association with attractiveness than with health (Steiger's $Z=3.48$, $p<0.01$; Figure 2.6). Log CRP was not significantly associated with any of the other variables ($p>0.1$), although the relationships were consistently negative.

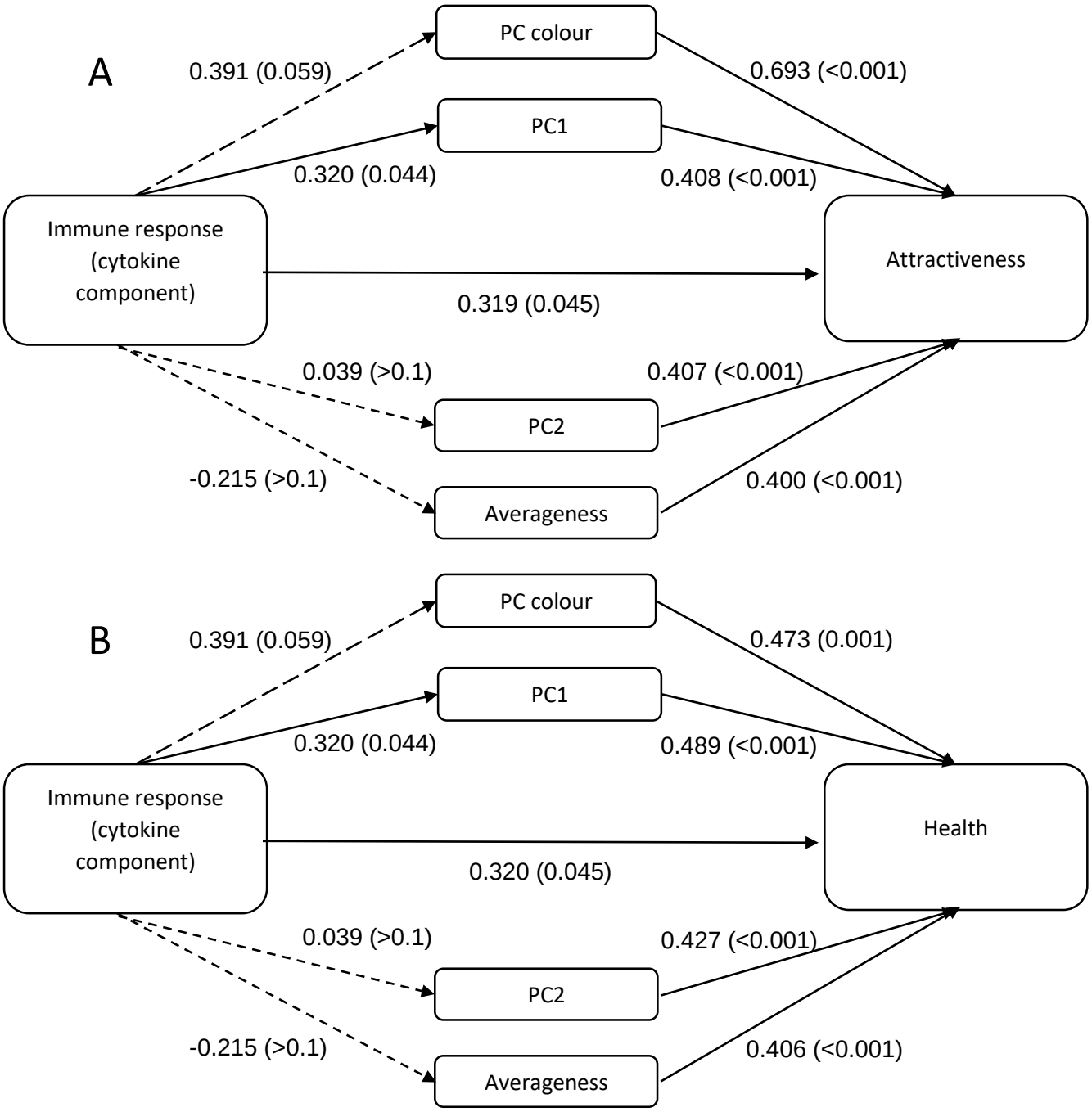

Figure 2.6: Pearson's Correlation coefficients for A) log facial attractiveness and B) perceived health. *Values indicate Pearson's correlation coefficients (r) with associated p-values in brackets. Short dashed lines indicate non-significant coefficients (p>0.05), long dashed lines marginal associations (p<0.1) and solid lines significant associations (p<0.05). PC1= masculinity, adiposity component; PC2 = symmetry, low adiposity component.*

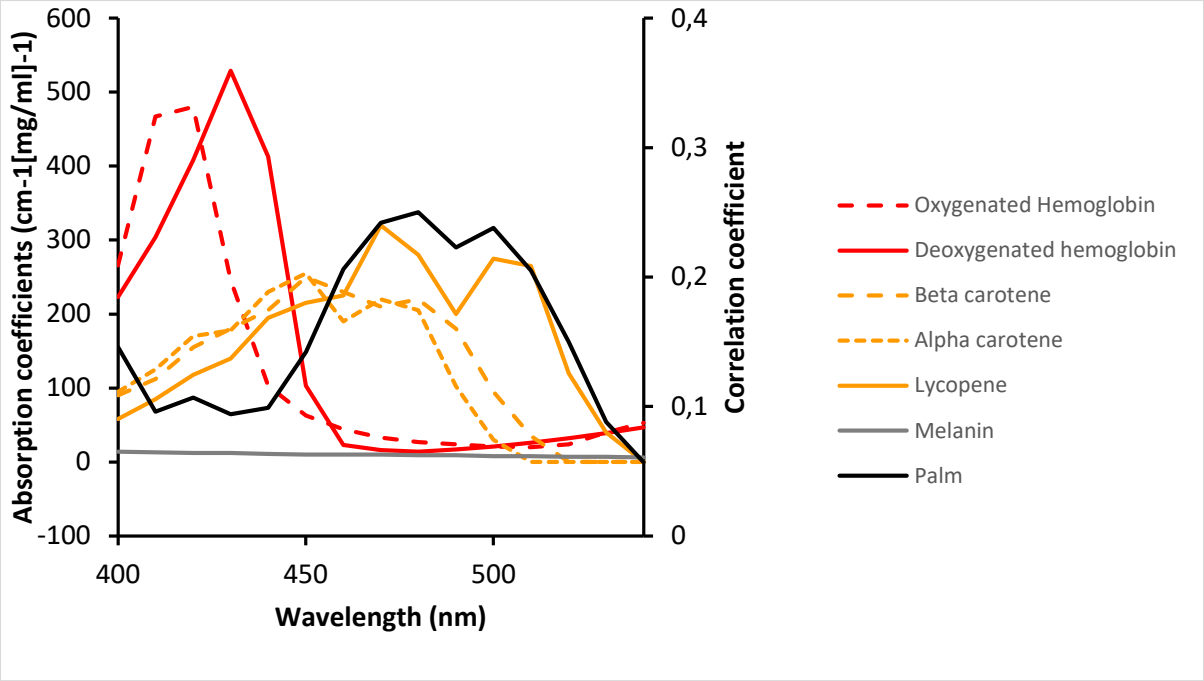

Figure 2.7: Spearman's correlation coefficients between participant's cytokine component and skin reflectance values in the <u>palm of their hand</u> plotted together with the absorption spectra of common human pigments: *Oxygenated hemoglobin, deoxygenated hemoglobin, β-carotene, lycopene and melanin. Correlation coefficient's were reverse scored (by subtracting from 0) to correspond to absorption rather than reflection spectra. Human pigment absorption spectra were obtained from Stephen, Coetzee and Perrett (2011). The correlations between the cytokine component and the spectral values closely followed the predicted spectral pattern for carotenoids, especially the carotenoid lycopene, but not the predicted spectral pattern for other human pigments.*

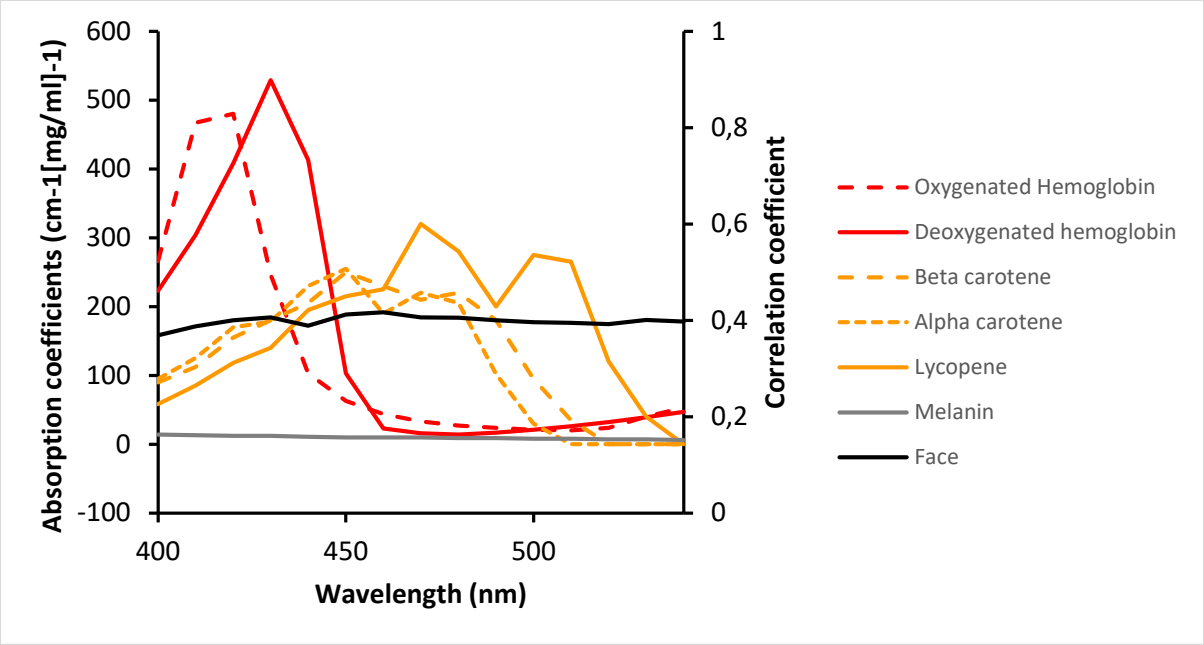

Figure 2.8. Spearman's correlation coefficients between participant's cytokine component and average facial skin reflectance values plotted together with the absorption spectra of common human pigments*. Correlation coefficient's were reverse scored (by subtracting from 0) to correspond to absorption rather than reflection spectra. Human pigment absorption spectra were obtained from Stephen, Coetzee and Perrett (20). The correlations between the cytokine component and the spectral values were closely associated with the predicted spectral pattern for melanin.*

2.3. Discussion

The aim of this study was to test the relationship between two direct measures of immunity (functional cytokine profile and CRP), overall facial appearance (attractiveness and health) and the five main facial cues (adiposity, masculinity, averageness, symmetry, skin colour) in African men.

From the results obtained, it is clear that African women rate African men with a stronger immune response (e.g. cytokine response before and after immune stimulation) more attractive and healthy than men with a weaker immune response. This finding is consistent with previous work, which found a significant positive association between Latvian men's antibody response after Hepatitis B vaccination and their facial and bodily attractiveness (48, 129).Interestingly, women with a higher

antibody response after Hepatitis B vaccination were not considered more attractive (49). Previous studies also found a significant positive association between HLA heterozygosity (an indirect measure of improved immunity) and facial attractiveness in young British and Australian men (43, 45), but not women (43, 46). Taken together, these findings demonstrate that men (but not women) with a stronger immune response are considered more attractive and healthy than men with a weaker immune response, irrespective of ethnicity. Previous studies, including some of our own, commonly studied the five main facial cues in isolation. Our results show that some of these facial cues are significantly associated. For example, masculinity and adiposity showed an intricate non-linear relationship, in that men who appeared more masculine also appeared heavier, but only up to a point, where after there was no additional increase in facial adiposity with increased masculinity. Symmetry was also positively associated with both masculinity and averageness, while averageness was somewhat associated with skin redness and yellowness (Table 2.2). Future studies could test whether these relationships hold in other populations. Studies that test these cues in isolation therefore run the risk of missing the bigger picture or attributing a relationship to one cue, while the relationship might also be explained (and even driven) by another correlated facial cue. A better understanding of these curvilinear relationships might also explain why positive relationships are observed in some studies and negative relationships in others.

Skin colour is strongly and positively associated with overall facial appearance (*e.g.* attractiveness and health) and marginally associated with immune response. The effect size for the skin colour-immune response relationship (r=0.391) was larger than the effect size for the significant masculinity, adiposity-immune response relationship (r=0.320), indicating that the marginal significance was likely due to the small sample size for skin colour values. These findings are consistent with previous work, which found significant positive associations between perceived skin health, HLA heterozygosity and facial attractiveness in British men (45). The three skin colour components, CIELab b* (yellowness), CIELab a* (redness) and CIELab L* (lightness), were highly correlated as in previous studies in African skin (155, 158). Nevertheless, the present study has shown that the cytokine component was more strongly associated with skin yellowness and lightness than redness. The correlation

between the cytokine component and spectral values of the skin was consistent with the expected pattern for carotenoids in the palm of the hand, especially the carotenoid lycopene. The carotenoid colouration was somewhat masked in the face, due to the presence of melanin, but significant associations between skin yellowness and immune response were observed in lighter faces. These results show that African men with stronger immune responses have yellower skin tones — consistent with what would be expected from a higher carotenoid content — compared to men with weaker immunocompetence. This relationship is, however, somewhat masked in men with darker skin tones.

Consistent with previous studies (153, 158), both yellowness and lightness played an important role in women's judgements of male facial attractiveness and health. This preference for a yellower, lighter skin tone can indicate a bias for lighter skin (Colourism; 157) or a preference for a yellow carotenoid colouration, which is more visible in lighter skin. Two lines of evidence favour the latter. First, we found a somewhat stronger association between attractiveness and skin yellowness than between attractiveness and skin lightness ($r\Delta$=0.008; although skin lightness was more strongly associated with perceived health [$r\Delta$=0.003]). This finding is consistent with previous work that also found a stronger association between facial attractiveness and skin yellowness, compared to skin lightness, in African men (158) and women (155); both $r\Delta$=0.047). Second, when asked to maximise healthy appearance in a previous study, participants increased skin yellowness more than lightness in both African and Caucasian skin (153). It is therefore more probable that the preference for a yellower, lighter skin tone is driven by a preference for yellow carotenoid colouration, which is more visible in lighter skin, than for a preference for lighter skin.

The masculinity, adiposity component was significantly and positively associated with cytokine response, attractiveness and health. Heavier, more masculine men therefore have a stronger immune response and are considered more attractive and healthy. At first glance this positive relationship between masculinity, adiposity component and cytokine response seem to contradict the previously observed negative association between antibody response and facial adiposity in Latvian men

(129). There is, however, one important distinction between the two populations: the Latvian cohort contained markedly less underweight men and more overweight-and-obese men (3% and 25% respectively; data not shown) compared to our African cohort (14% and 17% respectively). If it is assumed that normal weight men have a stronger immune response than under-and-overweight men *e.g.* (178, 189) one would expect a negative relationship in the Latvian cohort and a positive relationship in the African cohort (especially in the lower weight men within this cohort) which is what was found in the present study. Moreover, we showed that the masculinity, adiposity component is more relevant to underweight and lower weight men where an increase in adiposity is related to an increase in masculinity. This lack of underweight men in the Latvian sample likely also explains why Rantala *et al.* (129) observed a negative association between facial adiposity and attractiveness, while this and previous work observed a preference for intermediate weight in men (curvilinear relationship) (73, 183, 272).

Men with a relative BMI of 22.5kg/m^2 were considered optimally attractive and healthy, which is consistent with the optimal relative BMI preference of 23.6-24kg/m^2 in Scottish men (183). The masculinity, adiposity component dealt specifically with the left side of this curvilinear relationship, the positive association between facial adiposity and attractiveness in lower weight men. The positive masculinity-immune response component of the relationship is consistent with the previously reported significant positive association between masculinity and antibody response (129) and to some extend the weak positive association between masculinity and HLA heterozygosity (r=0.12; 7). Given the generally inconsistent relationship between masculinity and attractiveness (39, 256), it is not surprising that our positive masculinity-attractiveness relationship is consistent with some, but not all, previous studies on the topic.

The masculinity, adiposity component likely indicates facial features associated with muscularity in men, since: (i) facial adiposity and masculinity were positively correlated in under-and-normal weight men, but not in overweight men, which indicates an important role for muscle not fat; and (ii) African men generally have a higher percentage fat-free mass (mainly muscle mass) than other groups (*e.g.*

Caucasian men and women, African women), indicating that our under-and-normal weight men likely had a substantial amount of muscle (273-275). Furthermore, testosterone levels are known to increase muscularity and body weight (275, 276) and have been positively associated with facial masculinity (129, 256, 277). It is therefore likely that both facial masculinity and adiposity (in under-to-normal weight men) are associated with muscularity and testosterone levels. This is consistent with previous work which found a preference for cues to high testosterone levels in countries with a low Human Development index, such as South Africa (278). The masculinity, adiposity component was, however, more strongly associated with immune response than facial masculinity or adiposity by themselves, indicating that the masculinity, adiposity (i.e. muscularity) component might be a better estimate of the "androgen-mediated" trait proposed by the ICHH. Indeed, the significant relationships observed between this component and cytokine response is consistent with the ICHH's key assumption that "androgen-mediated" traits are associated with immune response (256). From the results obtained, it is proposed that there is indeed a link between immune response and an "androgen-mediated" facial trait in humans, but that the facial trait is *muscularity* (or strength (279) and not *masculinity* (i.e. the immune response hypothesis of male facial *muscularity*).

The symmetry, low adiposity component was significantly associated with facial attractiveness and health, but not with the cytokine component. Men with more symmetrical, skinnier faces were therefore judged more attractive and healthy, but they did not have a stronger immune response. These results are consistent with the non-significant association between symmetry and HLA heterozygosity in British and Australian men (43, 45) and the positive association between symmetry and attractiveness in other populations (39, 43). Whereas the masculinity, adiposity component dealt with the left side of the curvilinear relationship between facial adiposity and attractiveness (positive association in lower weight men), the symmetry, low adiposity component deals with the right side of the curvilinear relationship (negative association in higher weight men). Higher weight men in the upper normal-to-overweight range were considered less attractive, as in previous studies (73, 183). Higher weight men did not, however, have a weaker immune

response as expected from Rantala *et al*. (129) study, possibly because our population did not contain enough overweight and obese men.

Facial averageness was also significantly associated with attractiveness and health, but not with immune response. This finding is in line with previous studies, which also found more average looking men and women to appear more attractive (39, 43), but is not in line with the strong positive association found between averageness and HLA heterozygosity in Australian men (43). Averageness might be associated with certain aspects of immunity, but not others, or there might be population differences in the link between averageness and attractiveness. More research is needed to elucidate these relationships.

Similar results were observed for health and attractiveness due to the high correlation between the two variables, with one notable difference. The colour component was more strongly associated with attractiveness than health. This is somewhat surprising given the marginal association between immune response and colour, but our previous work also found that African participants rely quite heavily on colour, especially skin yellowness, when judging attractiveness in African men and women faces (155, 158, 159). We found no significant associations between CRP and any of the other variables, although the relationships were consistently negative, with some facial cues (such as skin colour and symmetry) showing larger effect sizes than the correlation between BMI and CRP in a large previous study (275). Interestingly, all the facial components, apart from the symmetry, low adiposity component, showed a much stronger association with cytokine response than BMI did. This finding is consistent with a growing body of evidence indicating a stronger association between facial features and some health outcomes, than more traditional measures of health, such as BMI (73, 280). Facial features therefore serve as a very useful predictor of health.

In conclusion, this study builds on previous work to firmly establish the link between facial attractiveness and immune response in men. The work also highlights the intricate relationships between different facial cues and the need to follow a more integrated approach when studying the link between health and facial appearance.

We show that two aspects of facial appearance are associated with immune response in African men. Men with a stronger immune response have marginally yellower and significantly more muscular appearing faces and women consider these faces to be more attractive and healthy. In other words, not only do women consider these men healthier, but they are actually healthy. Women also judge men with more symmetrical, average looking and skinnier faces more attractive and healthier, but these men don't actually have a stronger immune response. These findings shed new light on the ICHH and the "androgen mediated" traits associated with immune response in humans.

Chapter 3

Determining the link between HLA heterozygosity and facial appearance in an African population.

3. Introduction

Human mate choice has been an important topic for human behavioural ecologists for decades. Evolutionary theory predicts that individuals should choose partners that will provide both direct (ability to supply offspring with resources; 281) and indirect benefits ("high genetic quality" 1; 5). The "genetic quality" would be inherited by offspring and confer survival and reproductive advantages (281). According to Neff and Pitcher (282) "genetic quality" could be defined as the sum of two components: additive genetic effects or "good genes" and the non-additive genetic benefits, which are referred to as compatible genes (3). The additive genetic effects are made up of specific alleles that increase fitness independently of the rest of the genome whilst heterozygote advantage, inbreeding avoidance and epitasis contribute to non-additive genetic benefits (for review see; 3).

Studies suggest the HLA or linked genes could influence human mate choice in three ways: HLA disassortative preferences, Heterozygote advantage (HLA heterozygosity preferences) and HLA frequency dependent selection. Here we focus on HLA heterozygosity and frequency dependent selection. Heterozygote advantage states that heterozygous individuals have the ability to resist a broader array of pathogens (250). The extent of the benefit depends on the amount of overlap between presented antigen peptides (283). However, not all heterozygotes are equally resistant to disease, but as a rule heterozygotes are more equally resistant than homozygotes (283).

Rare allele advantage is the most popular form of the frequency dependent selection model. It states that new mutant alleles will have a fitness advantage because the pathogens have not adapted to them yet (225). However, once the alleles become more common the pathogens would adapt to them and their frequency would

decrease (284). According to the minority advantage hypothesis, once the old allele becomes rare enough the pathogens adapted to it will decrease. These alleles will then have the selective advantage and increase in frequency (285). One of the ways in which frequency dependent selection shapes HLA based mating preferences is the focus on the benefit associated with rare alleles. As previously mentioned, new rare alleles confer a selective advantage because very few pathogens are adapted to them. Due to the low frequency of these alleles they will more likely be present in a heterozygote than a homozygote, and thus favour a preference for a heterozygous mate which would be able to recognise a wider range of pathogens (47).

An alternative hypothesis for frequency dependent selection is that certain specific common alleles might be preferred because these common alleles increase the individual's ability to resist specific pathogens. Hill *et al.* (286), for example, showed that common HLA alleles were associated with improved resistance to malaria. A preference for common HLA alleles may also function to avoid mates with rare alleles that exhibit gestational drive. Haig *et al.* (287) defined gestational drive as an instance in which a maternal allele disfavours offspring during gestation that do not inherit it. Thus gestational drive may be a property of rare female alleles because as explained by Thornhill *et al*. (47) any driving effects of common alleles are likely to be successfully countered by the evolution of genes that prevent driving. A mate preference in males for females who possess common HLA alleles may function to avoid mates with alleles that show gestational drive and thereby reduce the likelihood of abortion of offspring (47).

Heterozygote advantage is likely the reason that the preference for more HLA heterozygous mates has evolved (288). Indeed, studies have reported significant associations between increased HLA heterozygosity of class I loci (A, B, and C) and delayed progression to Acquired Immune Deficiency Syndrome (AIDS) (289). In addition, heterozygosity has been shown to be somewhat heritable (290), thus a HLA heterozygous mate may also provide indirect benefits in terms of HLA diverse offspring with sound immunocompetence (288). HLA heterozygosity has been associated with perceived facial health and attractiveness in some but not all studies. Roberts *et al.* (45) presented facial images of British men to British women to judge. They found that the faces of HLA heterozygous British men were judged as healthier

and more attractive by British women than more HLA homozygous men. Similarly Lie *et al*. (43) used facial images of Australian men and women and estimated heterozygosity at 12 microsatellite markers (all in linkage disequilibrium with at least one HLA locus, including HLA-A,-B and –DBR1) and another 11 microsatellite markers on different chromosomes. They found that increased HLA heterozygosity, positively predicted male facial attractiveness in young Australian men (43, 44).

Coetzee *et al*. (46) genotyped African Tswana women for HLA-A and-B and showed the women images to African men. They found no significant relationship between HLA heterozygosity and attractiveness judgements in African women's faces (46). HLA heterozygosity was also not significantly associated with facial attractiveness in young Australian women (43, 44). Thornhill *et al.* (47) found no significant relationship between HLA heterozygosity and facial attractiveness judgments in men or women faces as judged by individuals of the opposite sex. They did, however, find a significant association between HLA heterozygosity and the attractiveness of men, but not women's, scent (17). The non-significant association between male facial attractiveness and HLA heterozygosity reported in Thornhill *et al*. (47) might, be explained by the wide range of ethnicities (Caucasian, Hispanic, African, American, Asian and Native American) and participant ages (18-54 years for men and 17-44 years for women) included in the study, which could have confounded the facial attractiveness judgements. Overall, HLA heterozygous men are generally judged more attractive than their homozygous counterparts, while HLA heterozygous women are not.

The relationship between the different facial cues and HLA heterozygosity has not been fully established. Roberts *et al.* (45) tested the relationship between HLA heterozygosity and apparent health judgements of skin patches in male faces, and found that the two are positively related, independent of facial shape information. In other words skin condition is significantly and positively associated with HLA heterozygosity in men. Lie *et al.* (43) found that HLA heterozygosity positively predicted male attractiveness, and specifically facial averageness, with averageness mediating the HLA heterozygosity-attractiveness relationship in male faces. In other words more heterozygous men's faces were considered more average and

attractive. Averageness was not, however, significantly associated with HLA heterozygosity in women faces (43). Facial symmetry was not significantly associated with HLA heterozygosity in either men or women faces (43, 44). Lie *et al*. (43) did not find a significant relationship between masculinity and HLA heterozygosity in Australian men, nor femininity and HLA heterozygosity in Australian women.

Taking the evidence that frequency dependent selection influences HLA based mating preferences (for review see; 240). Some researchers have investigated the relationship between common/ rare alleles and attractiveness or health, since specific rare alleles might confer fitness benefits under frequency dependent selection (46). Thornhill *et al.* (47) found that men significantly preferred the scent of women with more common HLA alleles over women with less common alleles, but the commonness of HLA alleles were not significantly associated with the male scent attractiveness. They also found no significant association between HLA allele commonness and facial attractiveness or facial symmetry in either sex (17). On the other hand, African Tswana women with more common HLA alleles reported significantly fewer cold and flu bouts per year, fewer illnesses in the previous year and rated themselves healthier than women with rare alleles (46), although allele frequency did not significantly predict facial attractiveness in African Tswana women when rated by male volunteers (46).

To my knowledge, no previous study has tested the relationship between HLA based mating preferences and facial appearance in African men. The aim of this study is, therefore, to test the relationship between two HLA based mating preferences (preference for HLA heterozygosity and a preference for rare / common HLA alleles), facial cues (*e.g.* masculinity, symmetry) and overall facial appearance (attractiveness and health) in African men.

3.1. Materials and methods

3.1.1. Ethics statement

This study was approved in writing by the ethics committee at the University of Pretoria (EC141002-083).

3.1.2. Participants

Seventy one African men (mean age=20.4, SD=2.8) were recruited from the University of Pretoria. The data was collected from the same participants used in chapter two, however an extra blood sample was collected for DNA extraction. Each participant provided written informed consent and completed a short questionnaire, including questions on gender and ethnicity. Full colour frontal facial photographs were taken with a Canon EOS 40D digital camera under standardized conditions. Participants were asked to maintain a neutral expression. The facial photographs were standardised for orientation and size using Psychomorph and other in-house software.

3.1.3. Image Ratings

Twenty African women (mean age =22.5; SD= 2.2) were recruited from the University of Pretoria to rate all the male facial photographs for attractiveness, health, symmetry, masculinity, distinctiveness and facial adiposity. The male facial images were presented in a randomised order on a computer screen. The females participants were asked to rate each image (How attractive is this person?; How healthy is this person?; How masculine is this person?; How symmetric is this person?; Please indicate this persons weight?; How distinctive is this face?) on separate 7-point Likert scales (1=very unattractive, 7=very attractive etc.) and for weight (1= very underweight, 7= very overweight). Distinctiveness was reverse coded for the analyses to indicate averageness. Each female participant provided informed consent and completed a short questionnaire including questions on age and gender.

3.1.4. Sample collection

Blood (5ml) was collected by a qualified phlebotomist using EDTA vacutainer® tubes (to avoid clotting) and transported at ambient conditions to the laboratory. The blood was used for DNA extraction following the Quick-DNA™ Universal Kit as per manufacturer's instructions (ZYMO Research). In short, a 200 µl sample was mixed with biofluid, cell buffer and proteinase K and incubated. The digested sample was bound using genomic binding buffer, cleaned and eluted into clean microcentrifuge tubes. The purified DNA quality and concentration was measured using a Spec Nanodrop; the 260/230 ratio, 260/280 ratio and the DNA concentrations in were recorded in ng/µl were recorded. The gDNA was analysed on a 1% agarose gel to ensure the DNA was intact. The DNA was diluted into 50 ng/µl solutions and stored at -20 °C.

3.1.5. SNP genotyping

Twelve Single Nucleotide Polymorphisms (SNPs) that were associated with a range of different HLA loci, some of which have been investigated in previous HLA mating preference studies (*e.g.* HLA-A,B, DRB1,DRB2 (44, 46, 116, 244) were selected for SNP genotyping (Table 3.1). The SNPs were included on a custom designed TaqMan® OpenArray® Plate (Applied Biosystems®) and quantified using the Quantstudio 12K flex system (Life Technologies) according to manufacturer's instructions for Genotyping (Applied Biosystems™ QuantStudio™ 12KFlex Real-Time PCR System user guide).

The genomic DNA (gDNA) 50 ng/µl samples that were of sufficient quality (260/230 and 260/280 ratios between 1.6 and 2) were thawed to room temperature and transferred to a 96-well MicroAmp® Optical Reaction Plate in the correct well positions according to the Genotyping-128 format . The OpenArray® Sample Tracker software was used to track the samples, from their position on the 96-well reaction plate, to their position on the OpenArray® 384-Well Sample Plate and was finally used to transfer the samples to the correct position on the TaqMan® OpenArray® Plate.

The samples were transferred from the 96-well reaction plate to the OpenArray® 384-Well Sample Plate; 2.5 µl of the gDNA sample (50 ng/µl) was transferred to the corresponding well on the OpenArray® 384-Well Sample Plate. 2.5 ul of Two-fold TaqMan® OpenArray® Genotyping Master Mix was pipetted into each well and the plate was sealed with foil to prevent evaporation and to protect the plate from light. The 384-well plate was gently vortexed then centrifuged for 1 min at 1 000 rpm and placed on ice for 1 hour. The TaqMan® OpenArray® Plate was thawed at room temperature. The Accufill™ system automatically loaded the samples from the OpenArray® 384-Well Sample Plate to the correct microscopic well of the TaqMan® OpenArray® Plate. The TaqMan® OpenArray® Plate was sealed with film using the QuantStudio™ OpenArray® Plate Press. The immersion fluid was then loaded into the TaqMan® OpenArray® Plate and the plate sealed. All samples and controls were genotyped in duplicate on the QuantStudio™ 12K Flex System.

3.1.6. Analysis

SNP quality control was performed using the Taqman® Genotyper Software. SNP calls were analysed for all samples. The assay call rates, minor allele frequencies (MAF) and Hardy-Weinberg Equilibrium p-values (HWE*) are reported in Table 3.1. One SNP (rs3129720) had a poor amplification rate (< 50%) and was discarded and not used for further analysis. An overall HLA heterozygosity score was calculated for each participant by summing all the heterozygous SNP genotypes for that participant. To calculate the overall “common alleles” score we first calculated which of the two alleles were most common in the dataset for each SNP and then summed all the common alleles for each participant. All further analyses were performed in SPSS version 23.

Table 3.1: Single nucleotide polymorphisms and minor allele frequencies of study participants*. * P values of the deviation from Hardy-Weinberg Equilibrium (HWE). MAF-minor allele frequency. ϕ indicates omitted SNP.*

GENOTYPE	SNP	ALLELE	MAF	HWE*
HLA-A; HCG8; HLA-J	*rs7758512*	T > G	0.201	0.906
HLA-A	*rs2571390*	A > C	0.184	0.063
HLA-A	*rs1052693*	C > T	0.388	0.879
HLA-C	*rs2853946*	A > T	0.434	0.553
HLA-C	*rs2524079*	G > A	0.428	0.763
HLA-B	*rs6940467*	A > G	0.320	0.221
HLA-DRB1ϕ	***rs3129720***	**T>C**	**0.130**	**0.297**
HLA-DRB2	*rs9271857*	G > A	0.490	0.060
HLA-DRB3	*rs477514*	G > A	0.140	0.593
HLA-DRB4	*rs9275572*	A > G	0.450	0.781
HLA-DRB5	*rs11677206*	C > T	0.300	0.729
HLA-DRB6	*rs9469220*	G > A	0.480	0.390

Prior to analysis, all variables were examined for accuracy of data entry, missing values, outliers, normality of their distributions and pairwise linearity (45). All values were normally distributed (two-tailed critical z-score = ± 3.29, p = 0.001), except facial attractiveness (skewness z=4.45). Log transformation successfully normalised the distribution (log attractiveness skewness z= 1.86, kurtosis z= -0.23). We conducted Principal Component Analyses (PCA) to reduce the number of correlated variables to an uncorrelated set. Pearson's correlations (2-tailed) were used to test the correlation between facial cues / components, overall appearance, overall heterozygosity and commonness of alleles. Next, we analysed the relationship between individual SNPs, the facial cues / components, overall appearance. To reduce the number of tests performed we (a) performed LD pruning, excluding single SNPs if two or more SNPs were in high LD (r^2>0.2), and (b) excluding SNPs that had too few (<15) individuals per category. Independent samples t-tests were used to test whether heterozygosity/ homozygosity at individual SNPs were associated with

overall appearance and the facial components, while analysis of variance (ANOVA) was used to test whether allele commonness at individual SNPs were associated with overall appearance and the facial components. If a significant result was observed between groups, post hoc comparisons were conducted to further identify differences within groups.

3.2. Results

As in chapter 2, figure 2.1 the quadratic relationship between adiposity and masculinity ($F=19.585$, $p<0.001$, $R^2=0.365$) was stronger than the linear relationship ($F=17.615$, $p<0.001$, $R^2=0.203$). The removal of one outlier further improved the significant quadratic relationship ($F=23.315$, $p<0.001$, $R^2=0.410$). Squared terms for facial adiposity and masculinity were therefore included in subsequent analysis due to these non-linear relationships. As in chapter 2 some of the facial cues were significantly correlated *(e.g.* adiposity and masculinity; $r=0.451$, $p=0.001$), thus a PCA was conducted on the facial cues: symmetry, averageness, masculinity, masculinity^2, adiposity and adiposity^2. Averageness had low communality (0.24) with the rest of the variables and was excluded from the PCA. The PCA produced two principal components with eigenvalue >1, which explained a cumulative variance of 90%. Masculinity (0.91), masculinity^2 (0.88), adiposity (0.76) and adiposity^2 (0.73) loaded highly on PC1 explaining 59% of the variance. PC1 is hereafter known as the masculinity, adiposity component, with higher values indicating more masculine, heavier faces. Symmetry (0.67), adiposity (-0.63) and adiposity^2 (-0.66) loaded highly on PC2 explaining 31% of the variance. PC2 is hereafter known as the symmetry, low adiposity component, with higher values indicating more symmetrical, skinnier faces. The colour values (lightness, yellowness and redness) were also highly correlated (Table 3.2). A PCA of the skin colour values produced one principal component with eigenvalue >1, explaining 94% of the variance. Lightness (0.96), redness (0.95) and yellowness (0.99) all loaded highly and positively on PC3, hereafter the colour component. Higher values for this component indicate lighter, redder and yellower skin tone. There was no significant association between the colour component, masculinity, adiposity component, symmetry, low adiposity component or averageness ($p>0.1$).

Table 3.2: Pearson's correlations between skin colour variables

	Redness	Yellowness
Lightness	.835***	.959***
	(44)	(44)
Redness	1	.931***
	(44)	(44)

$^{\phi}$p<0.1, *p<0.05, ** p<0.01, ***p<0.001. N in Brackets

3.2.1. HLA heterozygosity

Overall HLA heterozygosity was not significantly associated with any of the facial cue components (PC Colour; Adiposity, masculinity component; Symmetry, low adiposity component; or Averageness), attractiveness (Figure 3.1). As in chapter 2, we observed significant associations between all facial cue components (PC Colour; Adiposity, masculinity component; Symmetry, low adiposity component; Averageness) and attractiveness (Figure 3.1). HLA heterozygosity was also not significantly associated with perceived health (r= - 0.184, p=0.125), while all the facial cue components were significantly associated with perceived health: PC Colour (r=0.463, p=0.002); Adiposity, masculinity component (r=0.516, p<0.001); Symmetry, low adiposity component (r=0.434, p<0.001) and Averageness (r=0.440, p<0.001).

Table 3.3: Pearson's Correlation coefficients for HLA heterozygosity and facial attractiveness. *Facial attractiveness was log transformed.*

	HLA heterozygosity	Attractiveness	PC Colour	PC1	PC2	Averageness
HLA heterozygosity	1 (71)	-0.111 (71)	-0.025 (71)	0.109 (71)	-0.124 (71)	-0.123 (71)
Attractiveness		1 (71)	0.675*** (71)	0.406*** (71)	0.425*** (71)	0.438*** (71)

$^{\phi}$p<0.1, *p<0.05, ** p<0.01, ***p<0.001. N in Brackets

Next, we investigated the individual relationships between the heterozygosity of the eleven HLA-associated SNPs, overall appearance and the facial components using independent samples t-tests. Before conducting these tests we reduced the number of SNPs by (a) LD pruning (r^2>0.2) and (b) excluding SNPs that had too few (<15) individuals per category. One SNP (rs2853946) was removed because it was in high LD with another SNP (rs2524079) leaving 10 SNPs for analysis. There were no SNPs that had too few individuals per category. HLA homozygous men for SNP rs2524079 were judged significantly higher than their HLA heterozygous counterparts for perceived health; attractiveness; the symmetry, low adiposity component; the colour component; and averageness (Table 3.4). Similarly, HLA homozygous men for SNP rs477514 were judged significantly higher for the symmetry, low adiposity component than HLA heterozygous men at this SNP (Table 3.4). There were no other significant relationships (all p>0.05).

Table 3.4: Independent samples T-test between the individual SNPs (Homozygosity and Heterozygosity), facial cues (PC Colour; Adiposity, masculinity component; Symmetry, low adiposity component; and Averageness) perceived health and attractiveness.

		Homozygosity		**Heterozygosity**		**t**	**p**
		M	SD	M	SD		
rs2524079	**Health**	**3.841** (37)	**0.822**	**3.380** (32)	**0.555**	**2.760****	**0.008**
	Attractiveness	**0.367** (37)	**0.145**	**0.281** (32)	**0.106**	**2.785****	**0.007**
	Masculinity, adiposity component	-0.078 (37)	1.180	0.022 (32)	0.839	-0.407	0.685
	Symmetry, low adiposity component	**0.282** (37)	**1.042**	**-0.221** (32)	**0.952**	**2.080***	**0.041**
	Colour component	**0.442** (24)	**0.946**	**-0.353** (19)	**0.843**	**2.870****	**0.006**
	Averageness	**4.574**	**0.521**	**4.277**	**0.658**	**2.096***	**0.040**

		(37)		(32)			
rs477514	Health	3.693 (53)	0.770	3.322 (16)	0.694	1.724^	0.089
	Attractiveness	0.330 (53)	0.136	0.301 (16)	0.127	0.759	0.451
	Masculinity, adiposity component	-0.081 (53)	1.087	0.111 (16)	0.959	-0.634	0.528
	Symmetry, low adiposity component	**0.239** (53)	**0.969**	**-0.520** (16)	**1.016**	**2.714****	**0.008**
	Colour component	0.013 (33)	0.964	0.389 (10)	0.991	-1.075	0.289
	Averageness	4.462 (53)	0.555	4.275 (16)	0.754	1.085	0.282

^p<0.1, *p<0.05, ** p<0.01, ***p<0.001. M=Mean. SD=Standard Deviation.t= t test. p= p – value. N in Brackets

3.2.2. HLA common alleles

The overall number of common alleles (sum common alleles) was not significantly associated with any of the facial cue components (PC Colour; Adiposity, masculinity component; Symmetry, low adiposity component; or Averageness) attractiveness (Table 3.5). The groups have been classified as follows: Individuals with two common alleles are homozygous (always the same allele for that individual e.g. AA). Individuals with zero common alleles were homozygous (they have the same non-common alleles e.g. aa). Individuals with one common allele were heterozygous e.g. Aa. As in Table 3.3, we observed significant associations between all facial cue components and attractiveness (Table 3.5). Sum common alleles were also not significantly associated with perceived heath (r= - 0.018, p=0.884).

Table 3.5: Pearson's Correlation coefficients for the sum common alleles and facial attractiveness. Facial attractiveness was log transformed.

	Sum common alleles	**Attractiveness**	**PC Colour**	**PC1**	**PC2**	**Averageness**
Sum common alleles	1 (71)	0.058 (71)	0.121 (71)	0.182 (71)	0.069 (71)	-0.066 (71)
Attractiveness		1 (71)	0.675*** (71)	0.406*** (71)	0.425*** (71)	0.438*** (71)

$^{\phi}$p<0.1, *p<0.05, ** p<0.01, ***p<0.001. N in Brackets

Next, one-way ANOVAs were conducted to investigate the relationship between common alleles at individual SNPs, overall appearance, the facial cue components and averageness. Before conducting these tests we removed 7 SNPs because they had too few individuals per category (rs2853946, rs7758512, rs1052693, rs6940467, rs477514, rs11677206, rs946922), leaving 4 SNPs for analysis. There were significant effects of rs2524079 common alleles on attractiveness (F[2,66]=4.230, p=0.019), health (F[2,66]=3.603, p=0.033), Symmetry, low adiposity component (F[2,66]=3.986, p=0.020) and the colour component (F[2,40]=5.288, p=0.009). Further analysis showed that two common alleles for rs2524079 was primarily associated with yellower (F [2, 40] =4.160, p=0.023), lighter (F [2, 40] =5.014, p=0.011) "carotenoid" skin tone and to a lesser extent symmetrical, skinnier facial features.

Post hoc tests revealed that the mean score of two common alleles was significantly higher than the mean score of one common allele (*e.g.* heterozygosity) for attractiveness, the symmetry, low adiposity component and the colour component (Figure 3.1 A-D). In other words, men with two common rs2524079 alleles (AA genotype) were judged significantly more attractive, lighter, yellower and redder, more symmetrical and skinnier than men with one common allele. Men with two common rs2524079 alleles (M=3.80, SD=0.95) were also judged marginally healthier (p=0.067) than men with one common allele (M=3.40, SD=0.56; Figure 3.1 B). The

mean scores for zero common alleles (*e.g.* only rare alleles; GG genotype) were also significantly higher than the mean scores for one common allele for attractiveness (Zero common: M=0.35, SD=0.13; One common: M=0.28, SD=0.11) and health (Zero common: M=3.87, SD=0.75; One common: M=3.38, SD=0.56) and marginally higher for colour (Zero common: M=0.24, SD=0.77; One common: M=-0.35, SD=0.84; Figure 3.1 A-D). In other words, men with only rare alleles were judged significantly more attractive and healthier and marginally lighter, yellower, redder, symmetrical and skinnier than men with one common allele for rs2524079. Men with two common rs2524079 alleles were judged marginally more symmetrical and skinnier (M=0.65, SD=1.18) than men with zero common alleles (M=0.03, SD=0.88; Figure 3.1 C). Overall, men with two common rs2524079 alleles were generally judged more favourably than men with two rare alleles, albeit not significantly so. There was no other significant effect of common alleles on facial appearance (p>0.05).

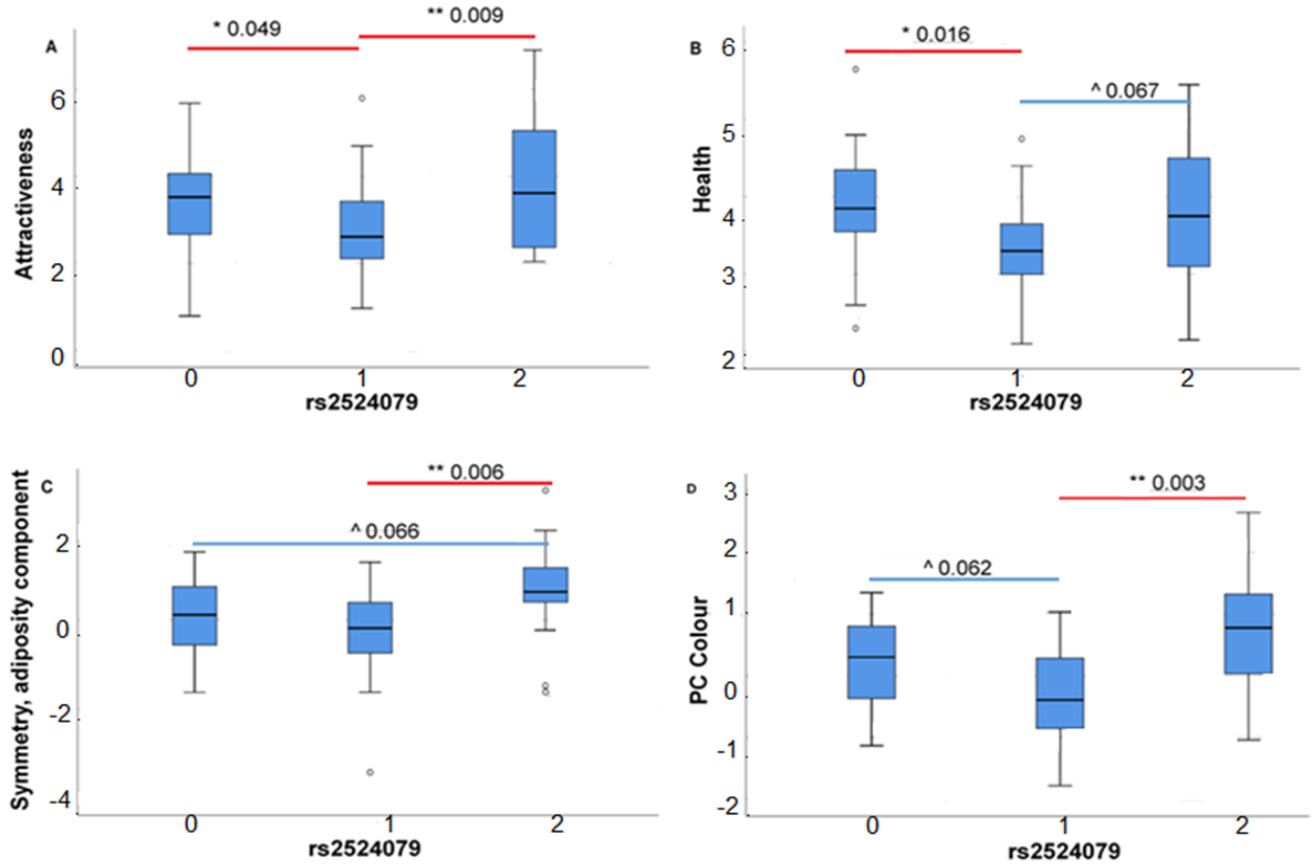

Figure 3.1: Box and whisker plots illustrating the difference in facial appearance between 0, 1 and 2 common alleles. ^p≤0.1, *p≤0.05, ** p≤0.01, ***p≤0.001. Error bars indicate standard error of the mean.

3.3. Discussion

The aim of this study was to test the relationship between two HLA based mating preferences (preference for HLA heterozygosity and a preference for common HLA alleles), facial cues (*e.g.* masculinity, symmetry) and overall facial appearance (attractiveness and health) in African men.

Overall HLA heterozygosity was not significantly associated with any of the facial cue components (PC Colour; Adiposity, masculinity component; Symmetry, low adiposity component; or Averageness) perceived health or attractiveness. These findings contradict the general positive relationship found between HLA heterozygosity and attractiveness in British (45) and Australian (43) men, but are consistent with the non-significant association between HLA heterozygosity and facial attractiveness in men from a heterogeneous (people of different ethnicities) sample (47), Australian women (43, 44) and African women (46).

There might be a number of plausible explanations for the discrepancies in the relationship between HLA heterozygosity and attractiveness in these different studies. Firstly, differences may be due to the sample sizes. Most of the previous studies had over 90 participants compared to our 71 participants, which might have resulted in our non-significant result. Secondly, HLA heterozygote advantage may be less beneficial in certain environments (*e.g.* where a few common pathogens cause most ailments) compared to other environments. For example, in African populations, most of the infectious diseases are caused by a few common pathogens (291). In such a scenario, one might expect an exceptional health benefit from alleles that confer resistance to these major pathogens. HLA heterozygosity is reported to contribute to general resistance to pathogens, but the specific alleles that confer specific resistance provide more protection under these conditions (46). Thirdly, the lack of agreement in the interrelation between facial cues and HLA heterozygosity might be because we may be overlooking other important cues to health for example diet and blood pressure. Fourthly, a trend in previous data suggests that women's preferences for the scent of HLA-heterozygous men may depend on the phase of their menstrual cycle (47), something which our study did

not investigate and should consider in the future. Fifthly, the nature of the alleles considered in our study represent a wider grouping of loci than those considered in previous studies (e.g. Roberts *et al.,* 45) genotyped participants at HLA-A,B and DRB1, whereas our study includes SNPs associated with several other HLA loci.

Overall HLA-associated allele commonness was not significantly associated with the facial cue components, perceived health or attractiveness, but we did find a significant association between allele frequency at the HLA- associated SNP rs2524079 and male facial appearance. Men with zero or two common alleles (homozygotes) for this SNP were judged more positively than heterozygotes, especially homozygotes with two common alleles. Homozygous men were judged as more attractive, healthier, marginally lighter, yellower and redder. Homozygous men were also judged as more symmetrical and skinnier. Our findings show that two common alleles for rs2524079 was primarily associated with yellower, lighter "carotenoid" skin tone and to a lesser extent symmetrical, skinnier facial features. A previous study by Thornhill *et al*. (47) did not find a positive relationship between allele commonness and symmetry. The findings for the preference for common alleles and HLA heterozygosity are inconsistent with the heterozygosity hypothesis (288) and heterozygote advantage (47) which posit that individuals prefer more heterozygous mates which produces heterozygous offspring that have sound immunocompetence against parasite types. Our findings show that African women prefer homozygosity at HLA associated SNPs (rs2524079 and rs477514) in African men.

Rs2524079 is a SNP found on the HLA-C region that have previously been associated with white blood cell counts (292). Nalls *et al.* (292) identified and replicated a significant association between rs2524079 and lymphocyte count in large Genome Wide Association (GWA) study involving > 30 000 participants. Lymphocytes are a type of white blood cell generated by the immune system to defend the body against cancerous cells, pathogens and other foreign substances (193). They play an important role in the immune system as they provide a means of immunity against antigens through both humoral (*e.g.* B cells creating antibodies) and cell mediated (*e.g.* T-cells which recognise various types of antigens) immunity

(193). These findings indicate that there are more adaptive hypotheses that explain the link between HLA genes, health measures and facial attractiveness besides heterozygote advantage (47). Our lack of evidence for heterozygote advantage, does not mean that heterozygote advantage cannot drive pathogen-related selection. This SNP for which significant effects were found is associated with HLA-C, which is not considered in previous studies, this SNP could be further investigated in British and Australian populations.

In conclusion our findings show that contrary to what is expected there is a link between some facial cues (symmetry, low adiposity component), health, attractiveness and preferences for homozygosity as opposed to heterozygosity in African men. We show that an HLA-associated SNP (rs2524079) which has been linked to lymphocyte count is positively associated with facial appearance in an African population. These findings therefore add a new perspective on the link between immunity and facial appearance in African men.

Chapter 4

4. Conclusions

Research into facial appearance in humans has evolved because of human preferences for healthy and fertile mates. Indeed, a positive association between facial appearance and health has been shown throughout the years, however the positive findings are far from consistent. Facial cues such as symmetry, averageness, sexual dimorphism, facial adiposity and skin colour have been positively linked with attractiveness and health (both perceived and actual) but the findings have not been consistent across ethnic groups and between men and women. I have provided several plausible explanations for the discordance between the findings on facial appearance and health/immunity throughout the thesis. One of the reasons for the discordances could be due to the way we measure health/immunity or more particularly the health measures traditionally used. Previous studies including some in our group have focused on BMI (73), blood pressure (73), antibody response (129) and mostly on self-reported health measures for example respiratory infections and antibiotics use (20, 21). Future studies could benefit from using both perceptual and quantitative measures. There is a need for more direct measures of health/immunity which is a shortcoming we have addressed through this work.

Facial appearance is thought to indicate immunity in humans, but very few studies have tested this relationship directly. Rantala *et.al* (129) tested the relationship between a direct measure of immunity (antibody response after Hepatitis B vaccination) and facial attractiveness in Latvian men, however antibody response only represents a small section of immune response. Other studies have tested this relationship indirectly by looking at HLA heterozygosity, which is not the only HLA based mating preference. According to our knowledge no study has ever tested the relationship between cytokine profiling and facial appearance. Cytokines give an overall idea of immune response as they function throughout the immune system.

The work in this thesis shows that the facial cues (symmetry, low adiposity, averageness and masculinity) are highly correlated and should not be studied in isolation. We also show that the facial cues are positively associated with health and attractiveness in African men. We show that African men with a high cytokine response are judged as more attractive and healthier, indicating that there is a positive link between immunity and facial appearance (health and attractiveness) in African men. We also show that cytokine response in African men is positively associated with a combination of facial cues (*e.g.* increased masculinity and adiposity, lighter, yellower skin tone). The "yellow bone" preference in African culture (*e.g.* people with a yellower skin tone are considered more attractive) could therefore be a preference for healthier partners. Cytokines should generally be released in inverse proportion depending on e.g. antigen levels. Our data shows that the cytokines were closely correlated which is in line with other literature that showed that some cytokines (IL-6, TNF-α) were correlated with each other in ulcerative colitis (UC) patients (295), syphilis patients (296) and healthy participants (297). This could be investigated further. These findings also shed new light on the "androgen-mediated" traits proposed by the immunocompetence handicap hypothesis (ICHH). In this chapter we proposed that facial muscularity serves as a better estimate of an "androgen-mediated" trait than the previously identified facial masculinity.

We also show that men's facial features in an African population reveal aspects of immunity, even better than other measures of health, such as body mass index (BMI). In chapter 3 we show that HLA heterozygosity is not associated with any of the facial cues, attractiveness and health in African men. Our results showed that contrary to what is expected homozygosity at three HLA associated SNPs was positively linked with facial appearance, symmetry and adiposity, skin colour and averageness. Our study also shows that there is a preference for allele commonness for one HLA associated SNP rs2524079. Individuals with two common alleles (AA genotype) were judged as more attractive, healthier, more symmetrical, skinner and were judged as lighter, yellower and redder. These findings are in line with the Nalls *et al*. (292) study which linked rs2524079 with lymphocyte counts and therefore immunity. Future studies should interrogate this SNP further in a larger population.

Our studies have some limitations which could be taken into consideration for future work. Firstly, our sample size was relatively small. We propose that cytokine profiling could be investigated using a biological sample (*e.g.* saliva) in future studies, which could be obtained non-invasively. This would encourage participation and increase sample sizes. Secondly, we did not test the influence of the media on facial cue (*e.g.* facial adiposity) preferences on health and attractiveness judgements. Studies have shown that women specifically internalise media messages about body ideals more than men do (293, 294), hence the judgements made by the women in our study could have been influenced by the media. Thirdly, in African population studies it might be helpful to compare health and attractiveness preferences across different ethnic groups (*e.g.* Tswana, Venda), although (a) the urban population is highly intermixed, and (b) Coetzee *et al*. (73) found that African participants couldn't assign ethnicity better than expected by chance. Fourthly, a recent study has shown that attractiveness judgements might be influenced by perceptions and experiences (133). In a South African population with a high crime rate, preferences for masculinity might be influenced by experiences. Thus it would be useful to take experiences into consideration for future studies.

In conclusion, our results indicate that different aspects of immunity might be indicated by different facial cues. Increased cytokine response after immune stimulation and two common alleles for the HLA-associated rs2524079 were both associated with increased facial attractiveness and healthy appearance, but different facial cues were involved in each. While increased cytokine response was primarily associated with more masculine, heavier facial features (i.e. muscular appearance) and marginally yellower, lighter "carotenoid" skin tone, two common alleles for rs2524079 was primarily associated with yellower, lighter "carotenoid" skin tone and to a lesser extent symmetrical, skinnier facial features. Our results also show that the relative contribution of different aspects of immunity might differ between different populations. While HLA heterozygosity has been positively associated with facial attractiveness in British and Australian men, specific common HLA-associated alleles seem to play a larger role in African men.

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