AN INVESTIGATION OF HEREDITY IN THE AETIOLOGY AND PATHOGENESIS OF CLEFT PALATE AND OF CLEFT LIP AND PALATE

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ETHICAL APPROVAL

All subjects involved in this study and, where appropriate, their parents gave informed consent for the data collection, for which Area Dental Ethics Committee approval had been obtained.

DEDICATION

To my wife Joanne

and

to the Mossey clan

throughout the world

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LIST OF ABBREVIATIONS

A	adenine
ABI	applied biosystems incorporated
ANOVA	analysis of variance
BamHI	restriction enzyme BamHI
BOG	biorthogonal grids
C	cytosine
CCA	conventional cephalometric analysis
cDNA	complementary DNA (made from mRNA)
CL	cleft lip
CL(P)	cleft lip with or without cleft palate
CLP	cleft lip and palate
CP	cleft palate
CPA	cleft palate with ankyloglossia
cRNA	complementary RNA (transcribed from DNA in a transcription vector)
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ECM	extracellular matrix
EDMA	euclidean distance matrix analysis
EFA	elliptical fourier analysis
EGF	epidermal growth factor
F13A	FXIII A, blood clotting factor
FEA	finite element analysis
g	gram

G	guanine
HLA	hyaluronic acid
kb	kilobase
kV	kilovolts
mA	milliamps
MEE	medial edge epithelium
MFT	multifactorial threshold theory
µg	microgram
mg	milligram
µl	microlitre
ml	millilitre
µM	micromolar
mM	millimolar
ms	milliseconds
MSX1	Muscle specific homeobox gene 1 (human)
Msx1	Muscle specific homeobox gene 1 (mouse)
nm	nanometre
OD	optical density
PC DIG	digitising software application (McWilliam, 1989)
PCR	polymerase chain reaction
pM	picomolar
RA	retinoic acid
RAR	retinoic acid receptor
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute

rRNA	ribosomal RNA
RsaI	restriction enzyme RsaI
SSCP	single strand conformation polymorphism
T	thymine
TA	tensor analysis
TaqI	restriction enzyme TaqI
TBP	total birth prevalence
TGF	transforming growth factor
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
tRNA	transfer RNA
TTR	transthyretin
UCLP	unilateral cleft lip and palate
χ^2	Chi-squared

List of abbreviations excludes:

(a) Cephalometric point or parameter abbreviations which are defined in Appendix B.

(b) Allele and genotype abbreviations which are defined in the text and appear in Appendix A.

ABSTRACT

Cleft lip with or without cleft palate (CL(P)) and isolated cleft palate (CP) are caused by primary defects in the fusion of craniofacial processes that form the primary and secondary palate respectively. CL(P) and CP are considered to be separate diagnostic entities and for both multifactorial inheritance has been proposed, although the precise roles played by genes, environment and chance are unclear; and in particular the nature and number of genes involved is not known.

The aim of this study was to try to identify parental characteristics (genotypic or phenotypic) which were associated with an increased risk of having a child with CL(P) or CP.

The parents of 53 children born in the West of Scotland over a five year period with non-syndromic clefts were investigated by cephalometric and DNA analysis.

Cephalometric analysis revealed that when compared to the male controls fathers of children with clefting tended to have statistically significantly smaller cross sectional areas of the cranium (c6, $p < 0.001$), smaller occipital subtenuce (c9/c10, $p = 0.042$), and more acute cranial base angles (N-S-Ba, $p = 0.036$). These fathers also had a smaller symphyseal area and total mandibular area, ($p < 0.001$ for both), while maxillary structures, palatal length (ANS-PNS, $p = 0.004$) and cross-sectional area of the maxilla were also significantly smaller ($p = 0.0015$). Using a stepwise discriminant analysis 83.3% of fathers and 82.6% of controls were correctly classified using these parameters.

Maternal differences compared to the female control were apparent in the cranial mandibular and anterior facial measurements. Cranial area (c6) was reduced in mothers ($p < 0.001$), parietal (c7) and occipital (c9/c10) subtenuce measurements were smaller ($p = 0.004$ and 0.0001 and respectively), whereas anterior cranial base length (S-N), and clivus length (S-Ba) were greater ($p = 0.016$ and $p = 0.033$ respectively). Also mandibular length, Cd-Gn was increased ($p = 0.011$) as was total face height (N-Me) ($p = 0.036$) when compared to the control. Using a stepwise discriminant analysis 95.1% of mothers and 98% of controls were correctly classified using these parameters.

The data was further analysed for parental craniofacial differences according to cleft type. A three-way analysis of CP, CL and CLP showed that not a single craniofacial parameter tested differed significantly between the CL and CLP groups, and

only four emerged as being statistically significantly different between CP and CLP. These were cranial area (c6, p = 0.049), mandibular area (s2, p = 0.047) and mandibular ramus length (Cd-14, p = 0.013); all of which were greater for the parents of children with isolated cleft palate (CP). Unfortunately the most significantly different of these parameters, the mandibular ramus length was subject to both systematic and random error in its measurement. Nevertheless this test was justification for the grouping together of the parents of children with CL and CLP for the remaining analyses in the study.

DNA samples from 76 parents and 19 probands were compared to a British control population (Holder et al., 1992) with respect to transforming growth factor alpha genotypes. The frequency of the TGFα/*Taq*I 1.7 kb allele (C2) in CL(P) parents (f = 0.13) was statistically significantly higher when compared to the control group (f = 0.04, p = 0.024). This increased C2 allele frequency in CL(P) **parents** is in accordance with previous studies on **probands** with CL(P). However, in addition the CP parental group showed a similar statistically significant increase in the C2 allele frequency (f = 0.15, p = 0.013) as did the small number of probands from both cleft categories (for CL(P) f = 0.17, p = 0.034 and for CP f = 0.19, p = 0.019). Calculation of relative risk (R) revealed that the probability of finding the C2 allele in the parental group is approximately four times higher than in the controls (for CP, R = 3.91 and for CLP, R = 3.38). The TGFα /*Bam*HI 10 kb allele (A1) frequency (f = 0.25) was also found to be significantly increased relative to the control (f = 0.13) in the parents of children with CP (p = 0.05). Conversely, the A1 allele frequency was slightly but not significantly reduced in the CL(P) parents (f = 0.08, p = 0.26). There was a highly significant difference in the A1 allele frequency between the CP and CL(P) parental groups (f = 0.25 and 0.08 respectively, p = 0.0075).

The chance of finding the A1 allele in the parents of children with CP is approximately doubled (R = 2.17) and for CL(P) is almost halved (R = 0.57) compared to the controls.

Parental genotype analysis revealed a cleft group and sex differential for the genotypes resulting from restriction enzyme digestion with *Bam*HI. Homozygosity for the A1 allele (A1A1) was unrepresented in any of the groups in the present study. The

frequency of the A1A2 genotype was increased among the CP parents (f = 0.50, p = 0.028) and decreased but not to the level of statistical significance among the CL(P) parents (f = 0.16) when compared to the control group frequency (f = 0.27, p = 0.23). This difference in the A1A2 genotype frequency between the two cleft groups was highly significant (p = 0.003).

Comparison of the genotypes resulting from restriction enzyme analysis with *Taq*I, of TGFα and PCR products (C1C1, C1C2 and C2C2) also showed statistically significant differences between the parental and control groups using a χ^2 analysis. Homozygosity for the C2 allele was unrepresented among the CP parents and the controls, and was present in only two CL(P) parents, both fathers. For this reason the C2C2 and C1C2 genotypes were combined for statistical analysis and a statistically significant increase in the C1C2/C2C2 genotype group was noted for both CP (f = 0.29, p = 0.022) and CL(P) (f = 0.26, p = 0.019) parental groups and for the cleft group analysed as a whole (f = 0.27, p = 0.006) when compared with controls (f = 0.08).

Further analysis revealed that the *Bam*HI/*Rsa*I genotype interaction is a potent discriminator between CP and CL(P). Applying a χ^2 test showed that compared to the controls A1A2/B1B2 predisposed to CP (f = 0.52 for the controls and 0.75 for the parents, p = 0.038) while A2A2/B1B2 tended towards predisposition to CL(P) (f = 0.47 for the controls and 0.86 for the parents, p = 0.067), and there was a highly statistically significant difference between CP and CL(P) for this *Bam*HI/*Rsa*I interaction (p = 0.0006). This analysis also highlighted the linkage disequilibrium between the *Bam*HI and *Rsa*I genotypes noted in previous studies. Using genotypic data alone, 68.3% of parents were correctly classified according to type of birth defect, CP or CL(P). The inclusion of gender did not influence the result, and so no statistically significant maternal/paternal effect can be attributed.

There was no correlation between parental genotype and any individual craniofacial measure, but a combination of genotypic and phenotypic data resulted in an improvement of the discrimination between CP and CL(P) over the use of either set of data alone.

Discrimination using the *Bam*HI/*Rsa*I genotype interaction combined with four craniofacial variables, the maxillary mandibular planes angle (MMPA), facial length

(S-Gn), cranial base length (S-N) and cranial width (Gla-CPo) resulted in 19 out of 25 CP parents (76%) and 30 out of 32 CL(P) parents (94%) being correctly classified. This is an overall rate of correct classification of 86% using the combined data.

Thus, the combination of phenotypic and genotypic data can improve the ability to predict parental predisposition towards CP or CL(P) beyond the predictive ability of either cephalometric or genetic data in isolation. The lack of correlation between these genotypes and the cephalometric parameters would suggest that other genetic loci are involved in the predisposition to CL(P) and CP and further analysis of other candidate genes using this approach would merit consideration.

CHAPTER 1

INTRODUCTION

1.1 CRANIOFACIAL MORPHOGENESIS

The earliest embryonic signs of the facial structures are apparent at about twenty two days with the appearance of the pharyngeal arches. The pharyngeal arches are bars of tissue that arise ventral to the hindbrain, each consisting of a mesenchymal core, partly derived from migratory cranial neural crest cells. They are covered externally by surface ectoderm and internally by epithelia of endodermal origin (Sulik and Schoenwolf, 1985). It is only the first and second pharyngeal arches in the rostrocaudal sequence which contribute to facial structures (figure 1). The first pharyngeal arch, although initially linear, becomes "C" shaped with differential growth. The rostral arm of the "C" becoming the maxillary process, and the caudal arm developing into the mandibular process (Streeter, 1945).

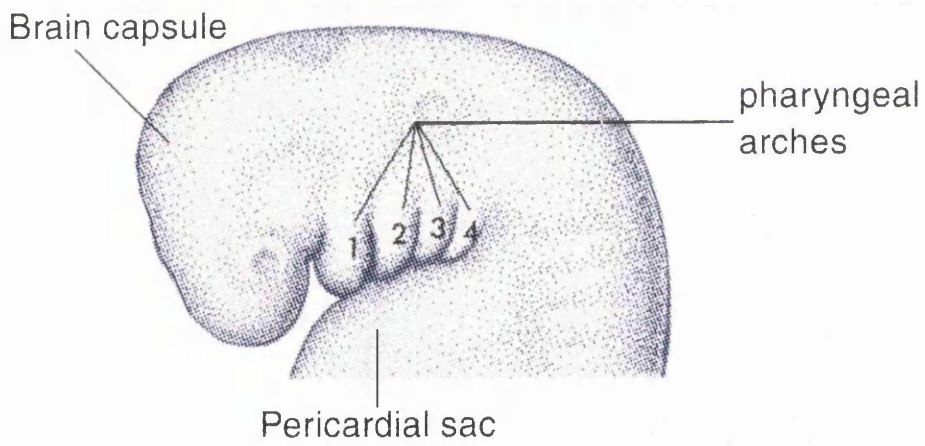


Figure 1. Lateral view of the developing embryo approximately 22 days post conception (adapted from Langman, 1981)

At around day 25 the stomatodeum or primitive mouth forms, bordered superiorly by the brain capsule, laterally by the maxillary and mandibular processes and inferiorly by the pericardial sac. Two to three days later the right and left mandibular processes begin to enlarge, grow medially between the stomatodeum and pericardial sac and merge in the midline to ultimately form the lower lip and mandible. At the same time the maxillary

processes begin to grow upwards and forwards beneath the brain capsule to form an ever-increasing portion of the upper jaw complex (Figure 2).

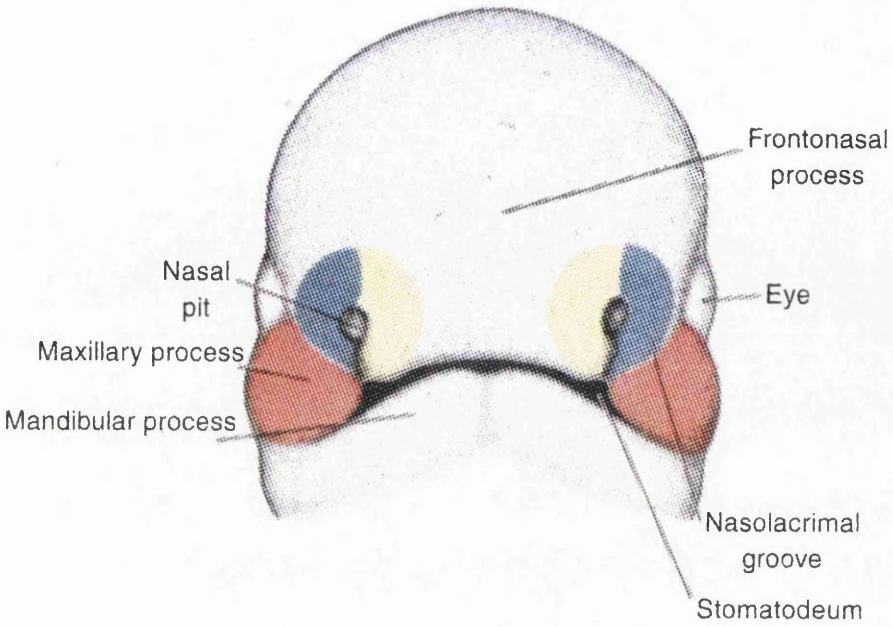


Figure 2. Frontal view of the craniofacial region of the developing embryo at approximately 28 days post conception (adapted from Langman, 1981)

1.1.1 Formation of the primary palate

The maxillary processes are widely separated from each other by the prominent medial and lateral nasal processes which are formed by proliferation of mesenchyme around the olfactory epithelium. During the fifth week the embryonic facial processes continue to swell as a result of proliferation of their contained mesenchyme and the ectodermal grooves or furrows that lie between these growth centres demarcate the facial processes. The elevating medial and lateral nasal processes surround each of the sinking nasal placodes during the fifth week creating deepening nasal pits that form the anterior nares (Figure 3).

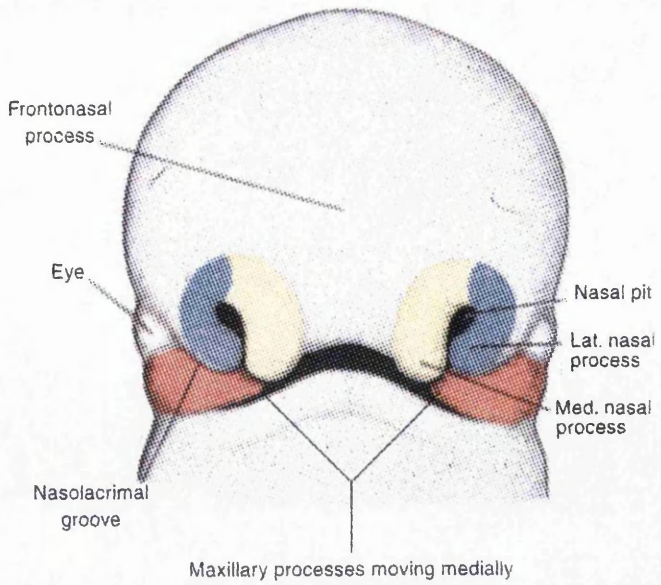


Figure 3. Craniofacial region of the developing embryo during the 5th week *in utero* (adapted from Langman, 1981)

The lateral nasal processes themselves form the alae of the nose, while the medial nasal processes which merge into a single globular process form the tip of the nose, the columella, the primary nasal septum and the entire primary palate (Sperber 1989) (Figure 4).

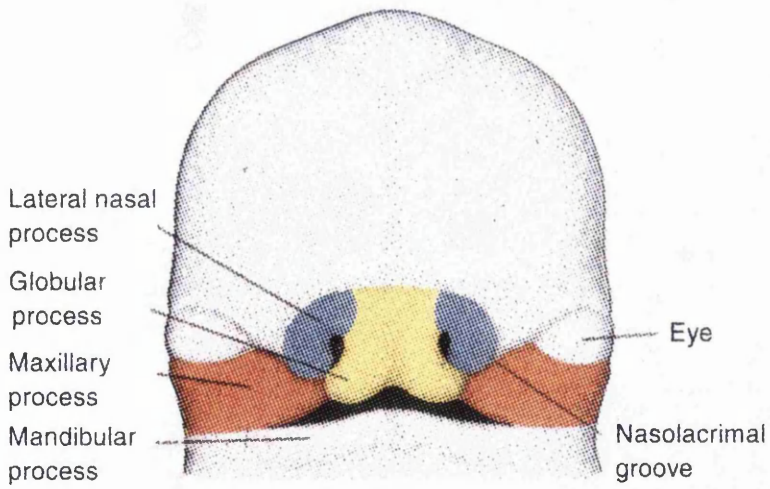


Figure 4. Merging of the medial nasal processes to form the globular process (adapted from Langman, 1981)

Previous controversy about the origin of the philtrum of the upper lip has been resolved by labelling of cells in the medial nasal and maxillary processes (Minkoff, *et al.*, 1984; Ferguson, 1993). These experiments show that the right and left maxillary processes completely overgrow the medial nasal and frontonasal processes to meet at the midline and form the entire upper lip (Figure 5).

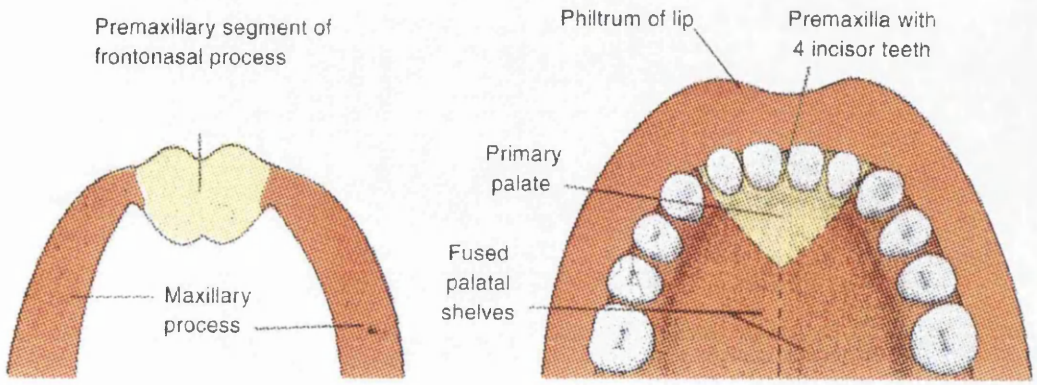


Figure 5. Formation of the entire upper lip by overgrowth of the maxillary processes (adapted from Langman, 1981)

Thus the philtrum and cupids bow of the upper lip are the result of the orientation of the insertion of the lip muscles and not a remnant of the embryological frontonasal process. In bilateral cleft lip and palate lip tissue seen on the abnormal premaxillary structure between the clefts arises from compensatory differentiation of the frontonasal process and is usually devoid of muscle.

1.1.2 Formation of the secondary palate

Towards the end of the sixth week, following primary palate formation, the oral cavity is roofed by the frontal process, walled by two lateral maxillary processes, floored by the merged first arches and occupied by the enlarging tongue. Separation of the stomatodeal chamber into separate oral and nasal cavities is first occasioned by the frontonasal and globular processes developing vertical and horizontal extensions into the chamber, the primitive nasal septum and primary palate respectively. Also during the sixth week bilateral extensions from the maxillary processes, the lateral palatal shelves,

begin to develop. Because the rapidly enlarging tongue completely fills the oro-nasal cavity the developing palatal shelves are forced to grow down into the only available space on either side of the tongue (Figure 6).

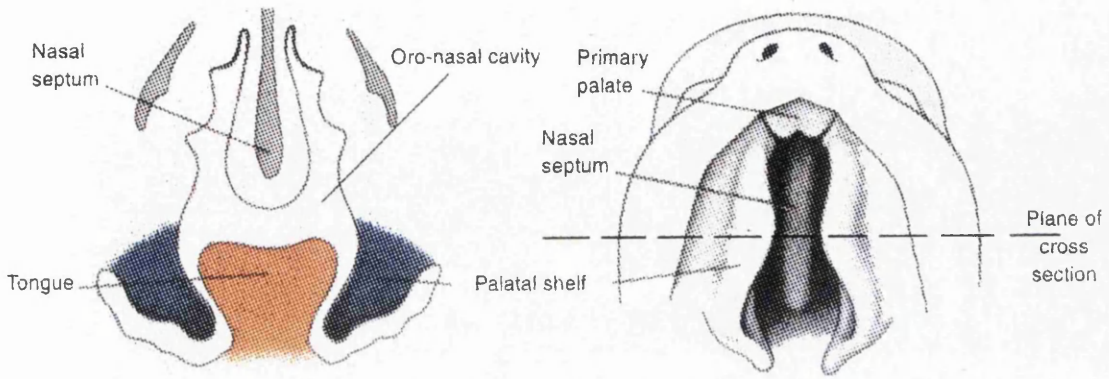


Figure 6. Diagrammatic cross section of embryonic head in the region of the secondary palate during the 6th week of intra-uterine life (adapted from Langman, 1981)

Development during the eighth week results in enlargement of the stomatodeum, enabling the tongue to drop into the lower part of the cavity and the shelves to elevate into the horizontal plane and approximate at the midline (Figure 7).

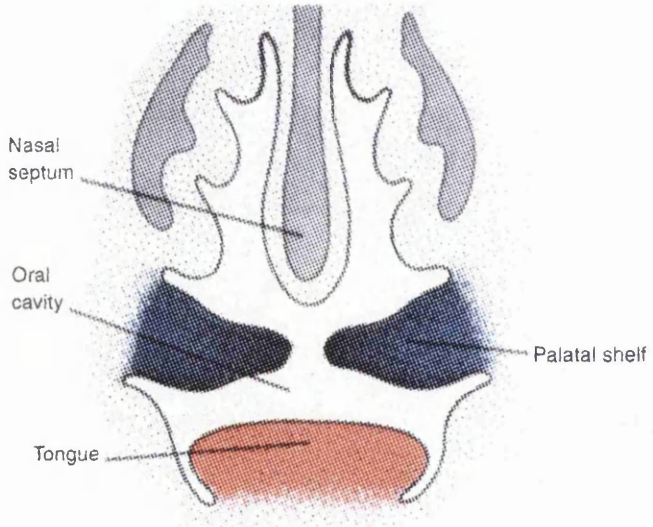


Figure 7. Elevation of the palatal shelves during the 8th week of intra-uterine life (adapted from Langman, 1981)

An interesting observation in respect of elevation of the palatal shelves is that the event occurs about one week earlier in the male embryo compared to the female embryo (Burdi and Silvey, 1969; Ferguson, 1987).

A number of factors, both intrinsic and extrinsic contribute to this shelf elevation mechanism which is a rapid embryological event. The intrinsic shelf elevating force comes from the synthesis of progressively more and more extracellular matrix molecules, principally hyaluronic acid which is capable of binding up to ten times it own weight in water. Hyaluronic acid producing cells concentrated in the acute angle between the palatal shelves and the maxillary prominences are in turn capable of causing the palatal shelf mesenchyme to swell and expand, and in combination with the contractile ability of the mesenchymal cells results in the flip up from a vertical to a horizontal position (Ferguson, 1988). Also during the 8th, 9th and 10th weeks *in utero* there is constant growth in head height but little or no growth in width (Diewert, 1985) which is conducive to elevation, and with foetal movements such as hiccups, mouth-opening reflexes and tongue muscle contraction beginning at around the 8th week the lowering of the tongue from its position between the palatal shelves is facilitated (Figure 8).

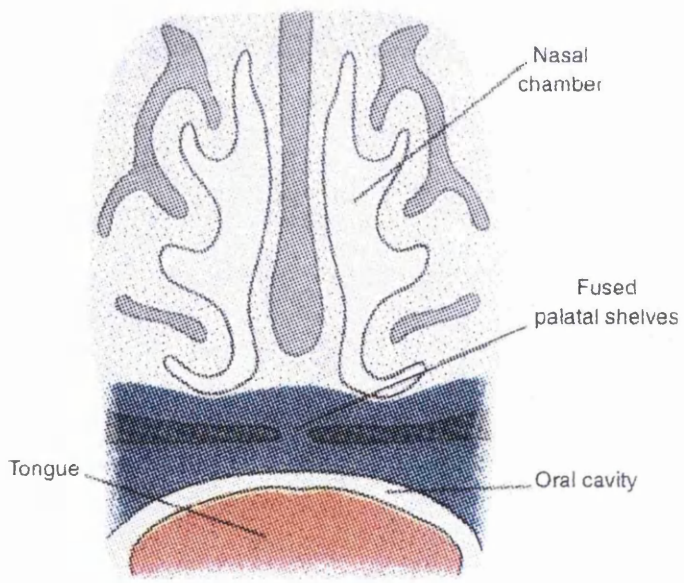


Figure 8. Contact and fusion of the palatal shelves and nasal septum during the 8th and 9th weeks of intra-uterine life (adapted from Langman, 1981)

When the shelves have elevated they make contact with the primary palate anteriorly, and with the lower portion of the nasal septum to form the definitive secondary palate. The medial edge epithelia fuse from the primary palate backwards to form an epithelial seam, and by a combination of cell death and cell migration this subsequently breaks down allowing the processes to establish mesenchymal continuity in the midline anteriorly with the primary palate and superiorly with the nasal septum.

At the same time as fusion occurs the palatal epithelium differentiates into nasal, medial and oral types, specified by the underlying mesenchymal cells. The epithelial-mesenchymal interaction is complex, and among other factors involved in the signalling, and therefore in normal palatogenesis are transforming growth factor alpha (TGFα), (see Section 1.1.4.3.) and transforming growth factor-beta (TGFβ). (see Section 1.1.4.2)

1.1.3 Embryonic development of specific craniofacial structures

This study will involve a detailed cephalometric examination of craniofacial morphology in the parents of children with cleft lip and palate. Previous studies have identified differences in mandibular, middle third and cranial base parameters and it is interesting to examine the embryonic development of these areas in particular.

1.1.3.1 The mandible

The growth of the mandible takes place by the sagittal growth of Meckel's cartilage. The formation of the cartilage begins in the central region of the mandibular prominences and continues both forwards and backwards in the right-hand and left-hand segments fusing anteriorly in the midline during week four of intra-uterine life. The growth of the embryonic cartilage takes place by means of cell proliferation, matrix volume increase and perhaps by the growth of cells.

In embryos of laboratory rodents, the growth of the mandible has been followed in a number of studies (Harris, 1967; Zeiler *et al.*, 1964; Hart *et al.*, 1969, 1972; Wragg *et al.*, 1970, 1972a; Shih *et al.*, 1974a; Diewert, 1976, 1980, 1982; Dostal, 1976; Jelinek and Peterka, 1977). However, the methods of cephalometric investigation have substantially differed.

In the period before horizontalisation of the palatal shelves the mandible grows more rapidly than does the nasomaxillary complex. During the 7th and 8th weeks the developing mandible gets below the primary palate. This is one of the factors contributing to the head elevation, to the increase of vertical dimensions of the oro-nasal cavity, and to the formation of the space necessary for palatal shelf horizontalisation. Diewert (1976, 1978) stresses not the importance of the absolute length of the mandible, but its relation to the primary palate. It seems evident that this key relation is important for normal palatal shelf horizontalisation and it may be influenced not only by the growth of the mandible but also by the length of the nasomaxillary complex, the cranial-base angulation, the growth of the palate and the position of the head as well.

1.1.3.2 The middle third of the face

A study on growth of the facial structures by Diewert (1985) showed that between the foetal ages of seven and nine weeks, the length of facial structures became approximately four times as long; whereas the height of the oro-nasal cavity doubled and the width was relatively unchanged. Linear measurements of craniofacial dimensions showed that the length of the mandible increased more than the length of the maxilla. The mandible became prominent relative to the maxilla at the time of palatal shelf elevation and remained prominent during the early foetal period. The elevated shelves are positioned in the new enlarged oro-nasal cavity space previously occupied by the tongue. The lifting effect is more pronounced in the anterior region of the palate, where the base of the maxilla becomes positioned above the height that the tongue occupied in the open palate at seven weeks. The base of the tongue becomes positioned lower (relative to the mandible) to create further vertical separation between the palatal shelves and the tongue.

This rapid sagittal growth of the nasomaxillary complex and the mandible during the last two weeks of embryonic development increases the prominence of the face relative to the anterior cranial base. In the nine-week foetal groups, the maxillary prominence relative to the cranial base expressed as angle SN to A point of

approximately 82° to 85° was similar to the normal angulation present post-natally (Diewert 1985).

Normal facial growth therefore tends to progressively separate the palatomaxillary processes from the tongue-mandibular complex as the nasomaxillary complex lifts upward and backward and the tongue is displaced forward with growth of Meckel's cartilage. In human cleft lip and palate, the clefting of the primary palate is believed to be the major factor in clefting of the secondary palate. (Burdi *et al.*,, 1967, Ross and Johnston, 1972). In mice with spontaneous clefting of the lip and palate, the wedged position of the tongue between the palatal shelves and the absence of head lifting appear to be the primary causes of shelf-elevation delay or failure and clefting of the secondary palate.

1.1.3.3 Cranial Base

A significant role in horizontalisation has also been attributed to changes of the configuration of the cranial base. In the mouse embryo, the angle between the anterior and posterior cranial base increases from approximately 130 to 180 degrees. This increase is partly due to the basioccipital cartilage remodelling, the growth of the nasal septum and the elevation of the anterior part of the head caused by the growing mandible and the tongue that become interposed between the primary palate and the anterior body wall. The augmentation of the cranial base angle may either be a consequence or the cause of horizontalisation.

A number of experiments have sought a connection between the cranial base angle changes, on the one hand, and induction of CP, on the other hand. Results of these are, however, equivocal. In the rat embryos with delayed horizontalisation and CP being induced by 6-aminonicotinamide (Diewert 1980), the degree of cranial base flexion has appeared similar to the controls. On the contrary, a straighter cranial base was found before horizontalisation in mice of the A/J strain that are more sensitive to the teratogenic action of corticoids (Diewert 1980).

1.1.4. Molecular aspects of palatal shelf development

There is an increasing curiosity about the molecular genetic aetiology of cleft lip and palate but as yet little is known about the molecular mechanisms involved. Recent studies in laboratory animals have implicated a number of extracellular matrix molecules in normal and abnormal palatogenesis and in the context of CP and CL(P) has led to further investigation into the pathophysiological role of TGFα and other closely related molecules, epidermal growth factor (EGF) and transforming growth factor beta (TGFβ). Each of these is discussed briefly below, and section 1.2.5 mentions their role in the aetiology of clefting.

1.1.4.1 Epidermal growth factor (EGF)

Epidermal growth factor (EGF) receptors are present in the palatal mesenchyme of mouse embryos from day thirteen onwards (Pratt 1984), and he also found that EGF is indispensable for cultivating palatal shelves in serum deficient media (Pratt *et al.*, 1980). Similarly EGF is indispensable for in vitro growth of palatal shelves on the extracellular matrix from cow endothelia (Grove and Pratt 1982). EGF inhibits the degeneration of palatal medial edge epithelia normally proceeding in vitro and promotes their hypertrophy and keratinisation (Hassell 1975). EGF receptors have also been demonstrated in human embryonic palatal shelves (Yoneda and Pratt, 1981).

All these data support the theory that EGF plays a physiological role in normal palatogenesis with experimental evidence suggesting a mediation role for EGF in epithelial-mesenchymal interaction. For instance, the effect of steroids on the differentiation of reproductive organ epithelia is mediated by their influence upon mesenchymal cells (Cunha, 1985). It has been observed on the other hand that the programmed cell death of the medial edge epithelia occurs even in palatal shelves deprived of mesenchyme three days before this morphogenetic degeneration (Tyler and Koch 1977). Finally in the study by Pratt (1984) certain similarities were found to exist between the embryonic EGF and the group of the so called transforming growth factors.

1.1.4.2 Transforming growth factor beta

Transforming growth factor type β, isoforms 1, 2 and 3 (TGFβ1, TGFβ2 and TGF β3) genes are part of an extensive family of polypeptide multifunctional cell regulators and have been suggested by Ferguson (1988) to be candidate genes in normal palate development on the basis of in vitro properties and immunohistochemical localisation. TGFβ1 and TGFβ3 may have a role in regulating cell growth in the palatal shelves. The timing of TGFβ3 expression as a putative growth inhibitor in the vertical shelves corresponds to the period when their linear growth ceases (Ferguson 1987). It is also known however that this is the period of maximal mesenchymal proliferation judged by counting mitotic figures in colchicine-treated embryos (Jelinek and Dostal, 1973) and [3H]-thymidine incorporation (Hassell *et al.*, 1974). It may be that delineated areas of proliferation adjacent to areas of inhibition may have a role in the dramatic processes involved in shelf reorientation.

FitzPatrick *et al.* (1990) carried out *in situ* hybridisation during murine palatogenesis to investigate the differential distribution of RNAs encoding the three related growth factors, TGFβ1, TGFβ2 and TGFβ3. The first appearance of TGFβ transcripts occurred at the late vertical shelf stage, with TGFβ1 and TGFβ3 being expressed in the medial edge epithelium, whereas TGFβ2 RNA is localised in the underlying mesenchyme.

TGFβ3 expression is predominantly expressed in the vertical epithelium facing the tongue and stopped abruptly on the oral side. In addition the epithelium of the nasal septum which is destined to fuse with the palatal shelves also shows a high level of TGFβ 3 expression. After elevation of the palatal shelves the medial edge epithelium (MEE) increases considerably. In the fused palate the TGFβ3 RNAs remain localised to the epithelial cells of the seam, but as the seam disrupts this expression is lost and the cells lose their epithelial phenotype by transformation into mesenchymal cells. The activation of TGFβ3 gene expression occurs 24-36 hours prior to palatal shelf elevation and fusion and ceases shortly thereafter. It could well be important in the MEE cell death mechanism (see section 1.2.5.1).

TGFβ1 is known to induce the synthesis of collagens and fibronectin (Roberts *et al.*, 1986; Ignotz *et al.*, 1987), tenascin (Pearson *et al.*, 1988) and chondroitin/dermatin proteoglycans (Hiraki *et al.*, 1988; Sharpe and Ferguson, 1988). Accumulation of the latter class of molecules is thought to be important in palatal shelf elevation by virtue of the rise in osmotic pressure resulting from hydration of the proteoglycan network (Pratt *et al.*, 1973; Brinkley and Morris Wiman, 1987; Derynck *et al.*, 1988a). In this respect it is interesting that high levels of TGFβ3 RNA are observed 24 hours prior to palatal shelf elevation.

The distributions of many extracellular matrix (ECM) proteins are fairly ubiquitous within the palatal mesenchyme. Two significant exceptions are collagen IX which appears on cell surface of MEE cells prior to shelf elevation (Ferguson, 1988), and tenascin which is localised beneath the medial edge epithelium prior to and during palatal shelf fusion (Sharpe and Ferguson, 1988). Since TGFβ1 is known to induce synthesis of both of these proteins (Sharp and Ferguson, 1988; Pearson *et al.*, 1988), it is a reasonable supposition that these ECM molecules may mediate some of the effects of TGFβs. The distribution of tenascin is particularly significant since the embryonic distribution of this molecule is almost completely correlated with the presence of epithelial TGFβ1 RNA (Chiquet-Ehrismann *et al.*, 1986; Lehnert and Akhurst, 1988; Akhurst *et al.*, 1990; Sharpe and Ferguson, 1988)

TGFβ2 RNA distribution during palatogenesis is in marked contrast to that of TGFs β1 and β3. Its predominant localisation in the mesenchyme would agree with the observations of Pelton *et al.* (1989) that mesenchymal expression of TGFβ2 might be important, not only in modulating the mesenchyme *per se*, but in supporting growth of the overlying epithelium via secondary events such as induction of TGFα. In this context, it is interesting that the TGFβ2 RNA distribution is asymmetric with respect to the nasal and oral regions. Differential concentrations of growth factors within the mesenchyme could contribute to the generation of regional heterogeneity of the overlying epithelium.

Further insight into the role of these isoforms may be gained by studying their localisation in the other developmental processes. Using mouse embryos Millan *et al.*

(1991) were able to report concomitant expression of two or more of the TGFβ isoforms in the epithelia of several developmental systems such as the salivary gland and the tooth bud (β1 and β2) RNA detected. These localisation studies show that each of the TGFβ isoforms are expressed epithelially at some point during murine and human embryogenesis. The association of these isoforms (TGFβ1 and TGFβ2) with epithelia overlying active mesenchyme in developmental structures beyond the palate strengthen the conclusion that they have a major role in the development of the palate.

1.1.4.3 Transforming growth factor alpha

The smallest form of transforming growth factor alpha (TGFα) is 50 amino acids long and shares 30% structural similarity with the 53 residue long epidermal growth factor (EGF), including the conservation of all six cysteines (Marquardt *et al.*, 1984). This sequence relationship and the presumed formation of three similar disulphide bridges by both molecules provide a molecular explanation for the interaction of the two growth factors with the same cellular receptor (Massague 1983). The peculiar structural features and the extreme sequence conservation between species suggest that TGFα has a biological function, but it still remains elusive.

Expression of the TGFα gene has been demonstrated in a variety of tumours, mostly in carcinomas, and consistently in squamous cell carcinomas and renal carcinomas (Derynck *et al.*, 1987). These observations have reinforced the belief that TGFα synthesis is associated with malignant transformation.

TGFα has however also been detected in nontransformed cells. During embryonic development in the mouse and rat, TGFα messenger RNA is transiently synthesised in several tissues, including the placenta, the developing kidney, the nasopharyngeal pouch, and the otic vesicle (Lee *et al.*, 1985). It is therefore suspected that TGFα plays a role in normal physiology and that its expression is not restricted to malignant transformation.

1.1.4.4 TGFα in cleft lip and palate

TGFα is synthesised during early foetal development, and since it competes with epidermal growth factor (EGF) for binding to the EGF receptor, it may function as a normal embryonic version of a family of EGF-related growth factors (Lee *et al.*, 1985; Twardzik, 1985) A transgenic mouse in which TGFα was over-expressed had epithelial hyperplasia in a number of tissues, thus demonstrating that TGFα is a potent epithelial mitogen (Sandgren *et al.*, 1990). Evidence for a role of TGFα is expressed in the epithelium of the lateral maxilla and throughout the palatal mesenchyme on embryonic day 12 but is sparse in the palatal epithelium. By day 13, staining is more intense in the palatal epithelium, and after palatal shelf elevation occurs, there is a marked increase in TGFα staining, particularly in the medial edge epithelium (Dixon *et al.*, 1991). The regional and temporal differences in staining for the transforming growth factor alpha suggests a role in normal palate development *in vivo*, particularly in degeneration of the midline epithelial seam. Precisely how this relates to a clefting disorder remains to be determined, but the genetic association observed between polymorphisms at the TGFα locus and clefting in humans may be important.

1.2 AETIOLOGY OF CLEFT LIP AND PALATE

The causes of congenital defects are generally divided into genetic and environmental. Purely genetic factors are considered to contribute to clefting in about 20% of cases with the genetic predisposition manifesting by the occurrence of a similar type of defect in siblings. Environmental factors have been established in approximately 10% of cases (Fara and Jelinek *et al.*, 1988). In the remaining 70% no single overwhelming factor of either genetic or environmental nature can be demonstrated and therefore the majority of congenital oral clefts are considered to arise under the influence of several factors. In the late 1960s, the multifactorial/threshold model MFT was advanced to explain the mode of inheritance of a variety of structural defects such as CL(P) and CP, which clustered in families but whose inheritance did not conform to mendelian laws. The model involved the concept of genetic liability or susceptibility to a given characteristic, governed by many different genes, and a threshold, determined by both genetic and environmental factors, (Fraser, (1970)). Individuals who lay beyond the threshold exhibited the phenotype, whereas those who did not were phenotypically normal. The model converted the normal distribution of a morphogenetic process within a population into the "all or none" expression of a structural defect.

The multifactorial/threshold model makes several predictions, specifically:

i) The defect in question will cluster in families;

ii) The risk for first-degree relatives of affected individuals (parents, siblings, and offspring) will approximate the square root of the population risk;

iii) The risk for second-degree relatives (uncles, aunts, half-siblings) will be sharply lower than the risk for first-degree relatives;

iv) The more severe the malformation, the greater the risk for recurrence;

v) The greater the number of affected family members, the greater the risk for recurrence;

vi) The risk for recurrence will be increased for relatives of the least affected sex, if gender differences are noted;

vii) Consanguinity will increase the risk.

In order to induce a developmental defect, the environmental stimulus needs to overcome a certain threshold determined by genetic factors, irrespective of what the nature of the latter may be. In those cases where the genetic predisposition is low (and therefore the threshold is high) only a strong exogenous impulse will be capable of overcoming normal morphogenesis. Conversely the greater the genetic predisposition, the weaker an external impact need be to produce a congenital defect.

Epidemiological studies have shown that the occurrence of orofacial clefts does not yield to simple Mendelian laws and the manifestation is conditioned by the accumulation of several genes whose action can be modified by extrinsic factors. Therefore unlike monogenetically determined diseases, the recurrence risk can be estimated in empiric fashion only i.e. on the basis of family studies. Such studies have shown that the recurrence risk in children of affected parents varies between four and 15% (Fogh-Andersen, 1942; Tolarova 1971, 1984).

Numerous surveys published in the 1970s and 1980s tested the "goodness of fit" of their population and family data to the predictions of the multifactorial model. Although many seemed to confirm the validity of the model, others tended to discount multifactorial inheritance in favour of a major gene effect because of clear discrepancies between the predicted and observed frequencies in recurrence risk for various relatives, rates of consanguinity, or gender effect of probands.

Recently, several investigators re-analysed previously published data sets with respect to a variety of alternative hypotheses, most of which assume the impact of a single major dominantly or recessively inherited gene. Although no single hypothesis has explained the observed data for cleft palate alone, several reviews involving multiple ethnic groups have supported a major single gene locus effect for CL(P), whereas others have suggested that either a few major genes or a mixed model (major gene plus multifactorial influences) produced better concordance with observed data, (e.g. Marazita *et al.*, 1986, Chung *et al.*, 1989, Hecht *et al.*, 1991).

Armed with the information that a single major gene may influence susceptibility to CL(P) in certain populations, several investigators have explored association between the

CL(P) phenotype and a variety of "candidate genes". These were chosen because the mechanism of action of the gene as assessed in animal models suggested a possible role in palatogenesis. The most intriguing and reproducible finding to date has been the association between CL(P) and one of two restriction length fragment polymorphisms (RFLPs) (different genetic forms) at the transforming growth factor alpha (TGFα) locus that maps to the short arm of human chromosome two at band p13 (Murray *et al.*, 1986), (discussed in more detail in section 1.2.5.3).

Recently, genetic linkage has also been demonstrated to one genetic form of the retinoic acid receptor gene (Chenevix-Trench *et al.*, 1992). This effect appears to be independent of the association with TGFα, suggesting that at least two major gene loci may be operative in nonsyndromic CL(P).

In summary, evidence is increasing that major genes predispose to nonsyndromic clefting in certain individuals and families. This information, however, has yet to be translated into clinical practice. Empiric recurrence risk figures will continue to be used for purposes of genetic counselling until the specific genes involved are identified and predictive testing is available.

1.2.1 The contribution of adverse environmental factors

It is not easy to demonstrate the contribution of environmental factors to human orofacial clefts. Retrospective methods of investigation which involve a search for the adverse factors only after the birth of the affected children introduce a remarkable memory bias. Unfortunately the vast majority of studies have been of this kind. Prospective studies, on the other hand, are much more reliable, but costly and time consuming, e.g., the collection of a sample of one hundred children with orofacial clefts prospectively would require a longitudinal examination of approximately fifty thousand pregnancies. Review of the literature on orofacial clefting does however reveal several significant environmental teratogenic factors. Alcohol is one such environmental agent which produces a characteristic craniofacial abnormality known as the foetal alcohol syndrome. This occurs in approximately one in one thousand live births and is characterised by microcephaly and a very typical appearance of short palpebral fissures,

short nose, long upper lip with deficient philtrum, small midface and small mandible (Jones *et al.*, 1983). This syndrome is thought to be caused by excessive cell death in the ectoderm, primitive streak and neural plate regions of the developing head end of the embryonic disc. Cell death at this early stage reduces the head field in size, such that the nasal placodes come closer together, giving rise to the characteristic facies later in the development (Ferguson, 1993). Other teratogens which have been implicated in the cause of cleft palate in man through epidemiological studies and also found to induce cleft palate in laboratory animals include corticosteroids, aspirin, diazepam and retinoids (Wilson, 1977). Teratogenesis in animals however does not imply teratogenesis in humans (Jelinek, 1984). Although cleft lip with or without cleft palate CL(P) and cleft palate alone (CP) may represent one feature of a number of recognised patterns of malformation attributable to environmental agents (alcohol, anticonvulsants, 13-cis-retinoic acid), isolated clefting (without associated structural and functional problems) has yet to be convincingly associated with prenatal exposure to a single substance. The higher incidence of acute and chronic infections, medication, endocrine imbalances, emotional stress and more frequently occurring pelvic X-ray examination are frequently mentioned to be characteristic of the mothers of cleft children. The agent that has been studies most extensively is maternal smoking. Although several well-designed, case-control studies have suggested between a two-to sixfold increase in the relative risk for clefts among smokers, other equally well designed investigations have yielded negative results. The fathers of children of clefts tend to be older on average than the fathers of normal neonates. However these teratogens may only have an effect where there is a parental genetic susceptibility. Further, cleft palate will only result if there is exposure to certain noxious environmental factors in the correct dose, in the correct combinations and at the correct time during pregnancy (Saxen, 1975). Glucocorticoids and retinoic acid are probably the two most extensively laboratory researched teratogens in respect of palatal clefting, and the findings may have some impact in human cleft palate and cleft lip and palate.

1.2.1.1 Retinoic acid in cleft palate

The retinoid group of molecules consist of retinol (vitamin A) which is an alcohol form that is metabolised to retinal (aldehyde form) and retinoic acid (acid form).

Although the retinoids are considered teratogenic at supraphysiological levels, their *in vitro* effects (Glick *et al.*, 1989) and the *in vivo* localisation patterns of retinoic acid (RA), (Thaller and Eichele, 1987), the binding proteins (Perez-Castro *et al.*, 1989) and the retinoic acid receptors RARs (Zelent *et al.*, 1989) suggest an important role for these molecules as morphogens. Retinoic acid in particular, has proven a very useful tool in the study of orofacial development. Cohlan (1953) first reported a specific pattern of craniofacial and limb anomalies in rats exposed to vitamin A in embryonic life. Subsequently many other studies have catalogued these anomalies in the chick (Tamarin *et al.*, 1977) and humans (Rosa *et al.*, 1986). Following marketing of the 13-cis form of retinoic acid for the treatment of severe acne, Rosa *et al.* (1986) reported 44 outcomes of pregnancies from women taking this drug during the first trimester. The pathogenesis of retinoic acid-induced cleft palate has been the subject of much study. Newall and Edwards (1981) have shown that it is possible to induce cleft palate in C57B1 mice by giving large doses only twelve hours prior to fusion. Abbott *et al.* (1989) have suggested that the aetiology of the retinoid-induced cleft varies with the embryonic stage. Those mice treated at gestation day 10 developed small palatal shelves that did not make midline contact. The medial edge epithelium (MEE) of these hypoplastic palatal shelves failed to undergo peridermal cell death and differentiated into oral type palatal epithelium. Those embryos treated on gestation day 12 showed no palatal growth inhibition with the palatal shelves making contact above the tongue. The MEE of these shelves, however, developed a nasal epithelial phenotype with the subsequent failure of fusion. It is of interest that the distribution of the epidermal growth factor (EGF) receptor is altered in the palates of retinoic acid exposed embryos (Abbott *et al.*, 1988).

The role of retinoic acid in mesenchyme proliferation, epithelial differentiation and growth factor expression is gradually emerging and this may clarify the mechanism of retinoid action in palatal clefting.

1.2.1.2 Glucocorticoids in cleft palate.

The role of glucocorticoids in embryogenesis has been under active investigation for many decades. One of the important characteristics of the corticoid induced cleft palate is the existence of remarkable inter-species as well as intra-species susceptibility. The role of glucocorticoid receptors in mediating the teratogenicity has indicated that the number of receptors and the particular metabolic pathways used by individuals may be important in determining susceptibility. Embryos of the highly susceptible A/J strain of mice contained many more glucocorticoid receptors in their maxillary mesenchyme than embryos of the resistant C57BL/6 strain (Salomon and Pratt, 1976; Katsumata *et al.*, 1982) In man, probands with cleft lip and palate have decreased numbers of glucocorticoid receptors and the protective effects of vitamin B6 have been explained by competitive binding to corticosteroid receptors (Yoneda and Pratt, 1982). Other vitamins, however, have been shown to be embryotoxic, such as vitamins A, D and E (Brandel *et al.*, 1985). There are still many unanswered questions regarding the mechanisms involved and possible interactions between vitamins and other drugs (Dostal and Blahova, 1986) and the relevance of experimental results to clinical practice remains unknown.

Although it is important to take account of the above, many studies mention the bias which is inherent in the retrospective character of many of these studies. There is uncertainty about the number of factors renounced, forgotten or simply omitted and in addition most of the embryos bearing developmental defects are eliminated prenatally. To supply direct proof of the toxicity of a particular substance to the developing human embryo is nearly impossible. In an instance where a drug has been administered during pregnancy it is impossible without appropriate controls to separate influences on development caused by the drug, and by the conditions for which the drug was taken. The present study sought to collect by questionnaire the details of the environmental teratogens which are thought to be significant in oral clefting. This data, however, is not discussed in the context of the aetiology of clefting with the main thrust of the present investigation being on the genetic aspects of clefting.

1.2.2 Epidemiology of cleft lip and palate

For over fifty years investigators have been carrying out birth prevalence studies on cleft lip and palate, CL(P), and cleft palate, CP, and from these reports the average birth prevalence of facial clefting in Caucasian populations is one per one thousand total births for CL(P) and one per two thousand total births for CP. Due to both the genetic and developmental evidence it is deemed justifiable to treat CL(P) and CP as separate entities (Fogh-Andersen, 1942; Kernahan and Stark, 1958). There is considerable phenotypic heterogeneity in the morphology of orofacial clefts but these have been categorised into three broad categories (Figure 9):

CL refers to cleft lip and/or primary palate which may be unilateral, (B, C) or bilateral (D).

CP refers to isolated cleft of the secondary palate (E).

CLP refers to cleft of the primary and secondary palate, which may be unilateral (F) or bilateral (not shown).

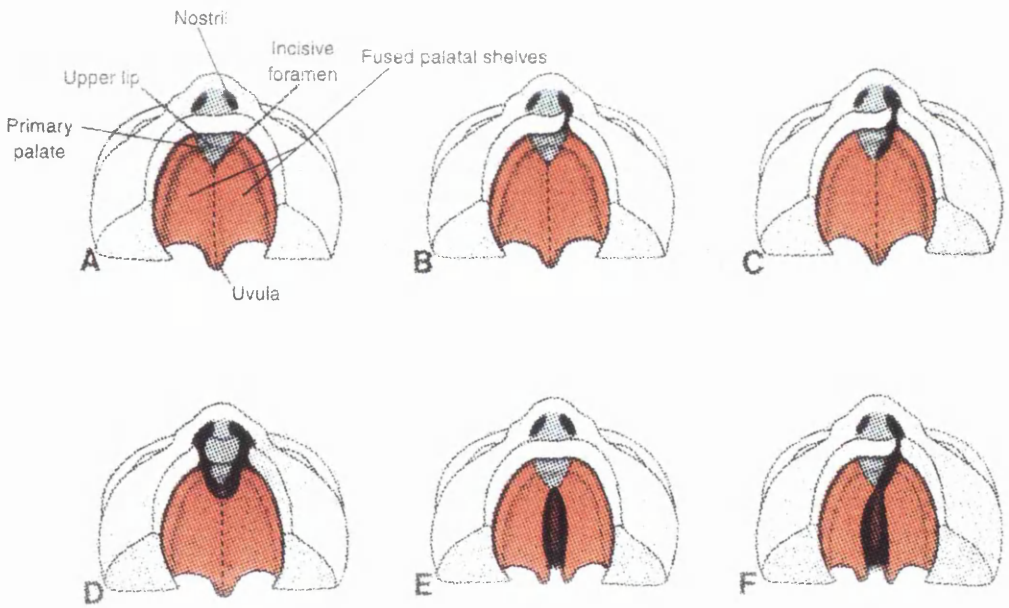


Figure 9 Diagrammatic examples of the variation in phenotype of orofacial clefts.(Adapted from Langman, 1981)
Figure A identifies normal palatal structures in a non-cleft palate. Figures B, C, D and F are all classified as CL(P) cleft lip with or without cleft palate.

Generally cleft lip alone or combined cleft lip and palate occurs more frequently in males whereas for isolated cleft palate the reverse is true. Also Fogh-Anderson (1942) reported that siblings of patients with CL(P) have a higher incidence of cleft lip, CL, and

of CL(P), but not of CP and that this homogeneity of defect occurrence also occurred in the siblings of cleft palate patients.

Significant racial differences in birth prevalence of oral facial clefts also exist (Table 1). The incidence of CL(P) varies from 2.1 per thousand in Japan and 2.7 per thousand in Canadian Indians to 0.4 per thousand in Nigeria and 0.42 per thousand in African Americans (Leck, 1972) with the geographical variation being less important than ethnic differences. Cleft palate alone (CP) however has a lower average incidence and is characterised by little variation in different racial groups: 0.41 per thousand in black Americans (Chung and Myrianthopoulous, 1968), 0.44 per thousand in Canadian Indian (Lowry and Renwick, 1969) and an average birth prevalence of 0.5 per thousand in European populations (Bonaiti *et al.*, 1982). Recent figures from Saudi Arabia reveal a remarkably low incidence of isolated cleft palate, 0.304 per 1000 live births (Borkar *et al.*, 1993), while in the West of Scotland a study by Womersley and Stone (1987) reported a remarkably high incidence of isolated CP, 0.81 per 1000 live births. The figure for the ascertained cleft population involved in the present study of 0.79 per 1000 live births (FitzPatrick *et al.*, 1991) confirms this observation. Only one other study found in the literature has recorded a comparatively high figure for the incidence of isolated CP in the population, that by Saxen and Lathi (1974) in the Finnish population with 0.86 per 1000 live births.

The racial differences in CL(P) birth prevalence are likely to have a genetic basis as shown by Ching and Chung (1974) in an extensive study from Hawaii. They showed that Japanese immigrants continue to have increased birth prevalence of CL(P) and by studying interracial crosses indicated that Caucasian-Japanese matings have intermediate birth prevalence. Leck (1972) also showed that the variation in birth prevalence of CL(P) between different ethnic groups living in the same areas is much greater than the variation among geographically scattered populations of the same ethnic origin. Racial differences in the prevalence of clefting are therefore thought to be independent of environment.

Table 1 Birth prevalences of facial clefts in previous studies.

Author, Year	Total Incidence (No. Cases)	CL	CLP	CP	Population
Fogh-Anderson, 1942	1·5 (286)	0·375	0·75	0·375	Danish
Neel, 1958	2·68 (171)	-	-	-	Japanese
Chung and Myrianthopoulous, 1968	1·82 (24)	1·34		0·48	European Americans
	0·82 (64)	0·41		0·41	Black Americans
Lowry and Renwick, 1969	3·17 (64)	0·25	2·45	0·44	Canadian Indians
	1·63 (737)	0·38	0·71	0·54	European Canadians
Saxen and Lathi, 1974	1·72 (599)	0·83		0·86	Finland
Bonaiti *et al.*, 1982	1·52 (646)	0·96		0·56	French
Womersley and Stone, 1987	1·56 (247)	0·21	0·54	0·81	West of Scotland
Jensen *et al.*, 1988	1·89 (678)	0·64	0·74	0·51	Danish
Borkar *et al.*, 1993	2·19 (137)	0·895	0·99	0·304	Saudi Arabia
* FitzPatrick *et al.*, 1992	1·53 (286)	0·27	0·47	0·79	West of Scotland

Total Incidence: This is given as the number of cases per 1000 total births.

(No. Cases): Refers to the total number of cases studied.

* Subjects used in present study came from this completely ascertained sample.

Various investigators have reported that birth prevalence figures provide evidence that isolated CP does not accurately fit the multi-factorial threshold model (Shields *et al.*, 1981; Carter *et al.*, 1982). Such a model includes both polygenic and environmental factors that tends to increase variation in incidence both geographically and racially. Instead evidence is accumulating which indicates that cleft palate may include both polygenic and monogenic types (Kurnit *et al.*, 1987; Fraser, 1989; Blanco *et al.*, 1993). Finally, a recent analysis of recurrence risk by Farrall and Holder (1992) has led to the proposal of an oligogenic model with as few as four loci involved, a conclusion arrived at simultaneously by FitzPatrick and Farrall (1993) when examining the West of Scotland data.

The differences in gender ratios within the facial cleft groups have proved to be more complicated, varying with severity of the cleft, the number of affected siblings in a family and racial origin. Studies on Caucasian populations for CL(P) indicate a consistent increase in the frequency of the anomaly in males with an average male/female ratio of two to one (Shapiro, 1976). In addition the male excess in the CL(P) group becomes more apparent with increased severity of the cleft (Fogh-Andersen, 1942). In Japanese populations there is a significant male excess in the CL(P) group, but not in the CL only group (Fujino *et al.*, 1963). In both races there would appear to be a slight excess of affected females in the CP group (Fraser, 1970; Shapiro, 1976). In African Americans there is an increased prevalence of isolated CL in females (Green *et al.*, 1964) but no overall significant gender differences in either CL(P) or in isolated CP. There is no generally accepted explanation for these gender differences, although gender differences in the timing of critical developmental stages in embryogenesis are thought to contribute to the aetiology. For example the fact that the elevation of the palatal shelves tends to occur approximately one week later in the human female than in the male, may play a role in the increased prevalence of cleft palate in females.

The consistent differences in laterality of the cleft lip group also remains unexplained. Two thirds of all cases of unilateral cleft lip (with or without cleft palate) have left sided defects regardless of gender, race and severity of defect (Fraser and Calnan, 1961)

1.2.3 Developmental pathogenesis of cleft lip and palate

In the context of clefting it is interesting that merging of the various prominences appears to be by a process of mesenchymal proliferation rather than ectodermal adsorption. The ventral sac epithelium (nasal fin) contains epithelial continuity between the nasal cavity and the roof of the mouth. Warbrick (1960) suggests that as contact is made between the ventral part of the maxillary process and the lateral side of the medial nasal process, an epithelium covering these processes is trapped and so gives rise to the fin. Streeter (1948) thought that the nasal fin was due to a proliferation of epithelium along the ventral fold of the nasal tract separating the maxillary and premaxillary growth centres. During the sixth week rupture of the ventral part of the nasal fin permits the mesoderm of maxillary process to blend with the mesoderm of the premaxillary centre of growth. It has been suggested that persistence of the ventral part of the nasal fin is responsible for cleft lip and anterior cleft palate (Warbrick, 1960).

At around 5½ weeks, just before fusion of the lip and primary palate the embryonic fusion lines between the maxillary, medial nasal and lateral nasal processes are clearly demarcated, all converging at one point see (Figure 5). Immediately before closure the lateral nasal process has a peak of cell division. This renders it very susceptible to teratogenic insults, and any disturbance in growth at this critical time can lead to failure of the closure mechanism (Ferguson, 1988). Cleft lip would result from failure along the embryonic fusion line extending medially between the lateral nasal, medial nasal and maxillary processes.

The factors that govern spatial patterning in the craniofacial region and therefore control palatogenesis are beginning to be elucidated. A number of morphogens, including retinoic acid, fibronectin, and a variety of growth factors, appear to play a role in this process; however, the neural crest cells seem to acquire their spatial programming while in the immediate proximity of the central nervous system, Wedden *et al.* (1986). Failure of fusion of the medial nasal process and maxillary prominence results in a typical cleft lip. Failure of formation of the frontonasal process results in a midline cleft lip and is usually the consequence of a severe defect in brain development.

The vertical to horizontal re-orientation of the palatal shelves occurs at a precise developmental stage around day 50 in man as they come into apposition above the tongue. The origin of the intrinsic force of elevation has been subject of much debate and is almost certainly multi factorial. Ferguson (1977) states that palatal shelf elevation is caused by an intrinsic shelf force generated by the hydration and expansion of palatal glycosaminoglycans, principally hyaluronic acid. This erectile force is directed by stout bundles of type one collagen which run down the centre of the palatal shelf and by the alignment of palatal mesenchymal cells which can themselves contract under the control of neurotransmitters. This build up of the intrinsic shelf elevating force occurs at a time when the embryonic head is growing constantly in height so that the position of least resistance for the expanding shelves is above the dorsum of the tongue.

After elevation the MEE cells of opposing shelves adhere to each other forming an epithelial seam. This epithelial seam accumulates lysosomal enzymes and undergoes programmed cell death resulting in mesenchymal continuity across the palate. Simultaneously the epithelium on the nasal surface differentiates into pseudostratified ciliated columnar cells and those on the oral surface into stratified squamous cells. Palatal epithelium therefore consists of three distinct regions (nasal, medial and oral) with different developmental fates. This precise epithelial differentiation is determined by the underlying mesenchyme via an inductive epithelial-mesenchymal interaction (Ferguson and Honig, 1984). Disruption of this interaction prevents MEE cell death and results in cleft palate. It is widely believed that in cleft lip and palate the cleft in the palate is largely a result of the preceeding cleft in the lip. The tongue becomes trapped above the cleft primary palate: this increases the resistance to shelf elevation and so a cleft in the secondary palate results (Trasler and Fraser, 1963).

A number of mechanisms including the following may be implicated in the pathogenesis of clefting:

I. Failure of shelf elevation at the correct time, due to altered synthesis of glycosaminoglycans and collagen, interference with neurotransmitter synthesis, or vascular problems e.g. haemorrhage. Shelf elevation may also fail because of postural moulding defects like Pierre Robin syndrome caused by contraction of the

foetal membranes due to insufficiency of amniotic fluid, trapping of the mandible beneath the sternum and consequent increases in tongue resistance.

II. Excessive head width due to asynchrony of the growth plateau in head width and the timing of shelf elevation. The gender differences in prevalence of cleft palate, being twice as common in females may be due to the fact that female palates elevate approximately 1 week later than males. Alternatively racial differences may have some bearing. Cleft palate is rare in Negroes, common in Caucasians and frequent in Mongoloids. This correlates with face shape, particularly facial width.

III. Failure of shelf fusion due to defective mesenchymal signalling of critical epithelial mesenchymal interactions; or defective medial-edge epithelial cell adhesiveness. Failure of medial-edge epithelial cell migration; or failure of differential gene expression could also prevent fusion of the palatal shelves.

IV. Postfusion rupture has also been proposed as a possible cause the evidence for which has been detailed over the years by Veau (1934), Steiniger (1942a), Scott (1955) and Kitamura (1966, 1991)

1.2.4 Inheritance of cleft lip and palate versus isolated cleft palate

The distinction between cleft palate (CP) and cleft lip with or without cleft palate (CL(P)) was first suggested by Fogh-Anderson (1942) in whose study gender ratios and familial clustering reflected an aetiological identity of cleft lip (CL) and CLP, whereas these phenotypes appeared to be aetiologically distinct from CP. He found that the male-to-female gender ratio for CL(P) was about 2:1 and the ratio in CP was about 1:2. Most family studies of facial clefts since have supported this aetiological distinction. In general CL(P) clustered within families, and CP clustered in other families. Alternate types of clefts did not occur within the same families at frequencies greater than that in the general population. Support for this segregation of cleft type comes from embryological evidence. In humans the lip develops between five and eight weeks *in utero* and the palate at about the ninth week. Fraser (1974) hypothesised that mechanical effects of cleft lip could secondarily cause cleft palate, which would account for the coincidence of the two types in families in whom cleft lip liabilities existed. On

the other hand, secondary palatal maldevelopment could occur after complete formation of the primary palate. This kind of disposition would lead to cleft palate only in some families.

Family studies have repeatedly shown that CP is genetically distinct from CL(P). CL(P) appears to have complex genetics. Curtis *et al.* (1961) estimated that the risk of recurrence in subsequently born children is 4% if one child has it, 4% if one parent has it, 17% if one parent and one child have it, and 9% if two children have it. Carter *et al.* (1982) followed up on the families of cases of CL(P), operated on at The Hospital for Sick Children (Great Ormond Street) London, between 1920 and 1939, to obtain information on the proportion affected of children and grandchildren. The probands were those who had survived, were successfully traced, and found to have had at least one child. They concluded that the most economical explanation of the findings is the multifactorial threshold model and that a single mutant gene is unlikely. Chung *et al.* (1986) analysed the genetics of CL(P), on a comparative basis in the Danish (Bixler *et al.*, 1971) and Japanese (Koguci, 1975) data. Japanese are known to have a higher population incidence of CL(P) and yet a lower recurrence risk among relatives than is true in Caucasian populations. Chung *et al.* (1986) concluded that the Danish data is best explained by a combination of major gene action and multifactorial inheritance. The major gene was thought to be recessive with a frequency of 0.035. Heritability was estimated as 0.97. On the contrary, the Japanese data could best be accounted for only by multifactorial inheritance with the heritability estimate of 0.77.

A complex segregation analysis of nonsyndromic CL(P) was performed by Hecht *et al.* (1991) in 79 families ascertained through a proband diagnosed at the Mayo clinic. In one analysis, the dominant or codominant mendelian major locus models of inheritance provided the most parsimonious fit. In another, the multifactorial threshold model and the mixed model were also consistent with the data. However, the high heritability (0.93) in the multifactorial threshold model suggested that any random exogenous factors were unlikely to be the underlying mechanism, and the mixed model indicated that this high heritability was accounted for by a major dominant locus component. Thus, the best explanation for the findings of the study was a putative major locus associated with markedly decreased penetrance for inheritance of CL(P).

Cleft palate as an isolated malformation behaves as an entity distinct from cleft lip with or without cleft palate. Curtis *et al.* (1961) estimated that the risk of recurrence in subsequently born children is about 2% if one child has it 6% if one parent has it, and 15% if one parent and one child have it. As for cleft lip with or without cleft palate, the genetics is apparently complex, but unlike population data relative to CL(P), data regarding CP alone rarely conformed to any model predictions suggesting marked heterogeneity in the study populations (Shields *et al.*, 1981; Carter, 1982 and Czeizel, 1984) and the probability of a greater environmental component. Shields *et al.* (1981) analysed family data on 561 Danish probands with nonsyndromic isolated cleft palate and concluded that neither a multifactorial threshold model nor a single major locus model is completely compatible with the distribution of cases. They proposed the existence of two classes of nonsyndromic cleft palate; (1) familial CP, which appears to have an autosomal dominant component, and (2) nonfamilial CP which also bred an increasing frequency of CP with time and maternal age and appears to be related to environmental factors. Carter *et al.* (1982) reported the findings in a series of patients who had been treated surgically for nonsyndromic cleft palate between 1920 and 1939. The authors suggested that the aetiology is probably heterogeneous with some families showing modified dominant inheritance. Other recognised disorders in which cleft palate alone is a feature are the Stickler syndrome, the Pierre Robin syndrome and the Van der Woude syndrome, but to date these have been unrevealing regarding aetiopathogenesis of the isolated defect. The Stickler syndrome is due to a mutation in the gene for type II collagen, the Van der Woude syndrome has been linked to markers on chromosome 1q32 (Murray *et al.*, 1990) and there is still even controversy about whether the Pierre Robin anomaly is a syndrome or a sequence.

At this time the exceptions to the dual entity assumption for clefts of the primary and secondary palates are insufficient to discount the overwhelming evidence of most family studies, which indicate their separateness. On the other hand, these exceptions point out clearly that the sharp distinction between the two groups of clefts may be too severe. In some contexts the genetic, physiologic and anatomic common denominators of midfacial maldevelopment may be segregating together within families and populations. These factors could predispose to both types of clefts. Single gene

abnormalities, teratogenic environmental factors, and gross chromosomal anomalies can produce clefting malformations.

The conclusion from these studies is that there is heterogeneity in both the pathogenetic mechanism of orofacial clefting and in the underlying aetiology. The aetiology is invariably multifactorial, the initiating factors innumerable and there is a varying contribution from genetics and environment. It is also noteworthy that the pattern of the clinical manifestation of clefting, whether CL, CP or CL(P) and whether syndromic or non-syndromic is no less variable than that of the aetiology.

1.2.5 Molecular genetics and clefting.

Familial recurrence risk analysis (e.g. Farrall and Holder, 1992) and complex segregation analyses (Marazita *et al.*, 1984; Marazita *et al.*, 1986; Chung *et al.*, 1986; Chung *et al.*, 1989; Hecht *et al.*, 1991) have concluded that a major gene (or genes) interacting with a polygenic background is consistent with the pattern of CL(P) recurrence in families. This has been strengthened by the association between CL(P) and a polymorphism in the TGFα gene (Ardinger *et al.*, 1989; Chenevix-Trench *et al.*, 1991; Holder *et al.*, 1991; Shiang *et al.*, 1991).

Eiberg *et al.* (1987) selected 58 pedigrees with nonsyndromic orofacial clefts from among a comprehensive collection of Danish cases for suggestiveness of autosomal dominant inheritance. Linkage with 42 non-DNA polymorphic marker systems was investigated. Both CL(P) and CP alone were, for the purpose of linkage analysis, scored as if being due to an autosomal dominant gene with complete penetrance. Linkage was found with clotting factor XIII A (F13A). The findings were taken to suggest that since F13A is located on the distal portion of 6p, a major locus for nonsyndromic orofacial cleft is also located in this region. Since both CL(P) and isolated CP pedigrees contributed to the positive score, it is possible that the locus on 6p carries two cleft alleles.

1.2.5.1 Epithelial/mesenchymal interaction in clefting

The processes involved in the disruption of the medial edge epithelium (MEE) have been the subject of much interest over the last two decades. For most of this time

the major mechanism in the disappearance of the epithelial cells was thought to be terminal differentiation, or "programmed cell death" with the removal of the cell remains by macrophages (Pratt and Martin, 1975; Greene and Pratt, 1976) Programmed cell death is a fascinating event in palatogenesis, with cessation of epithelial DNA synthesis estimated by the incorporation of [3H]-thymidine occurring 24-36 hours prior to fusion (Pratt and Martin, 1975). It is accompanied by a down-regulation in epidermal growth factor (EGF) receptors (Abbott *et al.*, 1988), and is not dependent on shelf contact in vitro. This lethal differentiation is specific to the medial edge epithelium; it is not seen in the oral or nasal components (Pratt and Martin, 1975; Tyler and Koch, 1975). However, little is known of the cellular events that cause this autolysis.

The precise molecular mechanism by which apoptosis is executed is unknown but it is known that all cells have the capacity to commit suicide, and require communication from neighbouring cells to prevent them doing so. The medial edge epithelium (MEE) cells of the palate undergo programmed cell death by epithelial-mesenchymal interaction allowing palatal shelf fusion.

Finally a relatively recent technology which has the potential to improve the understanding of protein function in cells and tissues is gene targeting and knockout. The search for function traditionally begins with immunocytochemistry to determine where and when the protein is expressed. The interpretation, that the protein is playing an important role at the sites where it is most prominently expressed, is almost universal. Recent gene knockout experiments (Sorriano *et al.*, 1991; Saga *et al.*, 1992; Shull *et al.*, 1992 and Erickson 1993) would suggest that these interpretations need to be re-evaluated. For example tenascin is a large extracellular matrix protein expressed in specific patterns in developing brain, cartilage, smooth muscle and in several tissues, including the palate, involving epithelial-mesenchymal interaction. Nevertheless Saga *et al.*, (1992) concluded following tenascin gene knockout that "mice develop normally without tenascin". Shull *et al.*, (1992) did likewise for TGFβ1, a protein which demonstrates powerful stimulatory and inhibitory effects on cell function and surprisingly the mice that were born had no apparent developmental defect. Of particular interest in orofacial clefting is the recent finding that gene knockout of TGFα produced mice that

had curly hair and curly whiskers but were otherwise apparently normal; while disruption of the TGFβ3 gene produces a mouse with cleft palate. These experiments have brought into question the importance of tenascin, TGFβ1 and TGFα in the embryonic palatal shelves despite their expression with such precise temporal and spatial regulation. The results of the aforementioned gene knockout experiments focus attention on the role of TGFβ3 in palatogenesis.

Another candidate gene for orofacial clefting which has recently been brought to the attention of craniofacial biologists through experiments in mice is the homeobox gene Msx1 (Muscle specific homeobox gene 1). Homeobox genes are a family of genes responsible for pattern regulation during development and they are thought to act by controlling the regional expression of other tissue differentiation genes. In vertebrates, the expression of Msx1 (and Msx2) is observed in many embryonic tissues which use epithelial-mesenchymal interactions during morphogenesis including the facial primordia, mandible and teeth. For example in the developing tooth bud, the dental epithelium is required to induce the underlying mesenchyme to differentiate into the dental follicle and dental papilla and Msx expression regulates this by mediating the inductive signals transmitted between the epithelium and mesenchyme.

In an experiment to determine the phenotypic consequences of Msx1 deficiency Satokata and Maas (1994) prepared mice lacking Msx1 function. They exhibited marked abnormalities in craniofacial development, including a complete cleft palate, a failure of tooth and alveolar bone development in the maxilla and mandible, and abnormalities of the skull, malleus, nasal bones and conchae. These Msx1 deficient mice provide a heritable monogenic model for cleft palate and raise the possibility that MSX1 (the human cognate) may be involved in related disorders of human craniofacial development. To date, however, no human craniofacial anomaly has been mapped to the corresponding region of the human genome, and none of the human pedigrees demonstrating autosomal dominant isolated CP appear to show linkage with MSX1 (Ferguson, 1994). It may be MSX1 mutations in man are associated with sporadic cases of cleft palate. Patients with non-familial clefting should undergo careful clinical evaluation to identify those with associated abnormalities of the teeth, nasal, frontal or parietal bones and the malleus of

the middle ear. The correlation between clefting and dental anomalies is well documented (e.g. Carretero-Quezada *et al.*, 1988), but in addition to oral examination, cephalometric and otolaryngological evaluation would also be required in suspected probands. Such patients could then be analysed for mutations in MSX1.

1.2.5.2 Rationale for molecular genetic investigation in clefting disorders

As the questions raised by epidemiological and clinical investigations on the inheritance of facial clefts become more clearly defined, it is of increasing importance to understand the mechanisms controlling mammalian palatogenesis at the cellular and molecular level. It is widely accepted that the field of molecular genetics offers the best chance of a breakthrough in the quest for the genetic contribution to clefting and interest has recently centred on the transforming growth factor alpha (TGFα) gene in the human genome. The genes in any disease process can be identified either directly by studying the expression patterns of candidate genes in normal and diseased tissue, or indirectly by linkage analysis or genotype/haplotype association. In this study the latter approach was taken and is based on the results of previous studies which have investigated the nature of structural alleles of the (TGFα) locus.

It has already been ascertained under segregation analysis that susceptibility to cleft lip and palate in the population under study is likely to be due to the action of a small number of genes (FitzPatrick *et al.*, 1990; Farrall and Holder, 1992). Ardinger *et al.* (1989) found an association between TGFα and the recurrence of cleft lip and palate, suggesting that either the TGFα gene itself or the DNA sequences in adjacent regions contribute to the development of cases of cleft lip and palate. A follow up study by Stoll *et al.* (1992) investigated the occurrence of the TGFα gene RFLPs both in patients with cleft lip and palate and in normal individuals. This revealed an association between the sub group with bilateral cleft lip and palate and a structural allele of the TGFα gene. Sassani *et al.* (1993) also studied allele and genotype frequencies at the TGFα locus in a mainly Caucasian CL(P) sample of 111 patients, 34.4% of whom had a bilateral defect. They found a significant association between the frequency of the TGFα C2 allele and CL(P). Moreover they found no significant difference in the C2 allele frequency between

cases with positive or negative family history. In view of the evidence from previous studies it was deemed appropriate that the TGFα site be investigated in the cleft lip and/or cleft palate population of the West of Scotland.

1.2.5.3 Analysis of transforming growth factor alpha polymorphisms

The gene for human TGFα is 70-100 kb (Derynck 1988) and is localised by *in situ* hybridisation on chromosome 2p13 (Tricoli *et al.*, 1986). Murray *et al.* (1986) and Hayward *et al.* (1987) have demonstrated RFLPs for the human TGFα locus, with the restriction endonucleases *Bam*HI (two alleles, 7.0 kb and 4 kb), *Rsa*I (two alleles, 1.5 kb and 1.2 kb) and *Taq*I (two alleles, 3.0 kb and 2.7 kb). By restriction with *Bam*HI, Qian *et al.*, (1991) has identified two-allele polymorphisms 10 and 7 kb, distinct from the two-allele *Bam*HI polymorphisms described by Murray *et al.* (1986). Because of the association of these two-allele polymorphisms with cleft lip and palate these TGFα RFLPs might be of interest as markers. The present study uses a PCR method coupled to restriction enzyme digestion to directly identify these TGFα polymorphisms.

1.3 THE MEASUREMENT OF FORM

1.3.1 Anthropometry

Attempts at measuring the face go back to the times of the ancient Greeks who used classical geometry to record facial dimension. Changes in shape of biological organisms, in two dimensional sections, were investigated by Thompson (1917) in order to quantify growth and differences between species. Differences in shape were represented using distorted co-ordinate grids. Hrdlicka (1920) described anthropometric measurement techniques using rulers, calipers and measuring tapes, and the same techniques have been used in the study of facial growth by many workers since then, such as Hellman (1929), Smyth (1932) and Meredith (1960). Anthropometric measurement, although quantitatively accurate, suffers from the serious drawback in that little descriptive or qualitative information can be derived from the measurements.

1.3.1.1 Stereophotogrammetry

Photographic methods have been used in anthropology to provide a qualitative record to supplement measurement records, but it was not until 1940 when Sheldon published his work on somatotyping, that the camera began to be used as a measuring instrument in the assessment of body physique. Tanner and Weiner (1949) modified and standardised this technique to such an extent that certain body dimensions could be measured to a degree of accuracy equal to that obtained in anthropometry. The face however is a difficult object to measure accurately due to the complex morphology, sensitivity of the eyes, tissue distortion on application of an instrument and posing error, especially in serial records. Stereophotogrammetry is a non-invasive technique which is capable of accurate three dimensional measurement of facial parameters without posing error or tissue distortion. A stereometric camera records a pair of facial photographs which can be placed in a plotting machine which reconstitutes a one to one visual image of the face. This may then be plotted as a contour map or digitised three dimensionally (Burke, 1984). Stereophotogrammetry has been used to measure facial growth in Pierre Robin Syndrome (Thalmaan-Degen, 1944), facial swelling (Bjorn *et al.*, 1954), facial

change in the edentulous (Haga *et al.*, 1964) and facial change due to facial surgery (Victorin *et al.*, 1971).

Many papers have suggested methods for the analysis of shape in two dimensions, but three dimensional analysis has been limited to few reported studies. A major advance in this field occurred in 1931 when Broadbent in America and Hofrath in Germany published comparable methods of recording standardised lateral and postero-anterior cephalometric skull radiographs. Broadbent (1931) suggested that these records be used quantitatively for three dimensional analysis, but very few studies have adopted this method. Herren (1961) proposed a new technique for recording radiographs of the bones of the face for three dimensional analysis, but no practical application of its use was reported. Savara *et al.* (1965, 1966) measured distances between bony landmarks in three dimensions from lateral and postero-anterior cephalograms with the aid of computers. Precision using all of these analyses depend on landmarks being readily and unambiguously identifiable.

1.3.1.2 Linear laser scanning

In principle stereophotogrammetry for measurement of surface detail of the face is a valid technique similar to cartography for analysing terrain in three dimensions. However, all the earlier differential geometric analysis of surfaces were limited by the enormity of computational requirement. Frobin *et al.* (1982) described the method which avoids the need for a unique body fixed co-ordinate system based on landmarks and the analysis performed allowed Frobin and Hierhoizer (1984) to identify in an objective way major features of the back. Moss *et al.* (1987, 1988) described a method of analysing facial shape using laser technology. A laser beam is fanned into a vertical line using a cylindrical lens. The line is projected onto the patient's face and is then viewed obliquely by a video camera. Camera output is digitised and a digital comparator suppresses superfluous signals. The patient is rotated under computer control and the distortion of the laser line as it illuminates the face is recorded at every 2.8° of rotation, except over the central portion of the face where it is recorded at 1.4°. The data is stored in computer memory and the approximately 20,000 co-ordinates on the facial

surface are derived. A patchwork of triangles is constructed from these to represent the facial surface with a precision of 0.5 millimetres. Coombes *et al.* (1988) applied a surface classification scheme described by Besl and Jain (1988) to the facial surface. This enabled them to produce an objective shape characterisation which correlates to a good degree with clinical observation. In 1991 Coombes collaborated with Moss to describe a three dimensional mathematical description of facial shape with the information readily available from the laser scanning system. This method enabled computer prediction of surgical outcome and comparison of facial shape in three dimensions before and after surgery.

1.3.2 Cephalometry

The conceptual basis of cephalometric radiology is that;

i) radiologic data is acquired using a methodology by which repeated measurement will produce the same data and

ii) that the acquired data is used to describe, analyse and/or compare craniofacial size, shape or both.

The most commonly used radiographic method for such data acquisition in clinical orthodontics involves a method of repeatable orientation of the patient's head in a cephalostat to produce lateral and postero-anterior cephalometric radiographs.

1.3.2.1 Conventional cephalometric analysis

The conventional cephalometric approach (CCA) for description of craniofacial morphology uses distances, angles and ratios. A wide variety of cephalometric analysis systems has emerged, each using different combinations of linear and angular measurements. Statistical analysis can then be carried out on various sets of measurements of interest. The main advantages of the conventional metric approach are its simplicity of use and the ease of interpretation and statistical analysis of results. Moreover the almost universal use of conventional cephalometric analysis for both clinical and research purposes makes communication and inter-centre comparative studies possible.

Despite its widespread use many workers over the years have pointed out the limitations of conventional cephalometric analysis for the description and study of craniofacial morphology. Some of these problems have been discussed by Steiner (1959), Krogman and Sassouni (1957), Bjork and Solow (1962), Walker (1967) and Enlow (1968) to name but a few. All of these workers pointed to the fundamental difficulty being the inherent complexity of describing craniofacial morphology using a numerical model. They recognised that biological form and growth cannot be adequately described using the conventional metric approach, but they were unable to offer viable alternatives. In more recent years Bookstein (1978), Moyers and Bookstein (1979), Lestrel (1980), Moss (1985) and Lele and Richtsmeier (1991), all presented their reasons for believing that conventional cephalometry is an inappropriate tool for morphometric analysis. Each of these workers however did so in the context of an alternative model which they believed would circumvent some of the difficulties with CCA. Four of these new methods,

I) Tensor analysis,

II) Finite element analysis,

III) Elliptical Fourier Analysis, and

IV) Euclidean Distance Matrix Analysis

will be reviewed here.

The limitations of CCA can be summarised as follows:

a) Landmark identification error.

Landmarks are often referred to as homologous points, the minimum criterion is that, given a single definition, it can be consistently and reliably located with a measurable degree of accuracy on all forms considered. In conventional cephalometric analysis the homologous points are either anatomic points, material points (e.g. implants) or constructed points, the latter usually being projected onto a cephalometric outline by manipulation of the available anatomic or material points on the radiograph. CCA has been criticised in that a) imprecise definitions and b) variations in the orientation of the subject in space will tend to vary the

precise location of some landmarks, e.g. pogonion and B point which are defined as the maximum convexity and maximum concavity respectively on the external bony outline of the mandibular symphysis.

b) Orientation and Superimposition

Standard two dimensional cephalometric analysis is dependent on the use of a line between two points, or a linear outline of a cephalometric structure to enable orientation of a radiograph or of successive radiographs for location of other landmarks or comparison of morphology. Moyers and Bookstein (1979) claimed that the malplacement of the orientation plane may arise from faulty positioning of the subject, inappropriateness of the landmarks for that subject or because of disproportionate growth or asymmetry. Cephalometry is thus susceptible to a form of mismeasurement in which error in the orientation is propagated to effect the positions of all orientation dependant landmarks.

c) Lack of surface and internal change data

It is widely recognised that CCA does have limitations when researchers attempt to measure shape. Moyers and Bookstein (1979) point out that the use of conventional cephalometric points reduces a curvi-linear biologic form to a geometric collection of straight lines. Lestrel (1989) maintains that landmarks cannot represent shapes and CCA being a metric numerical model is unable to extract a significant percentage of the informational content that resides in complex morphological forms.

Longitudinal cephalometric studies are often used for analysis of growth or shape changes. Lestrel *et al.* (1986) showed that the use of angular measurements as indicators of shape measures may not be adequate. He pointed out that angles tend to cover large aspects of the craniofacial morphology and the shape lying within an included angle is not even being measured. Others such as Medawar (1950) point out that angles are only appropriate for the measurement of regular geometric objects and cannot be used as a numerical description of the shape of complex or regular forms. Lestrel quotes the example of investigation of change of the cranial base angle, basion-sella-nasion. He showed that substantial angular

changes occurred in spite of the fact that the cranial base itself was stable and did not change appreciably. Further investigation showed that nasion was the major site of these angular changes rather than the cranial base itself. The inherent cause of this misnomer is that the sella-nasion line cuts across morphological structures and the point nasion (N) which is the fronto-nasal suture is not in fact on the anterior cranial base which terminates at foramen caecum.

d) Subjectivity

If the investigator in the study chooses the parameters which he considers to be relevant measurements, an unavoidable subjective element enters the analysis. The example quoted previously with the choice of nasion as the anterior landmark of the anterior cranial base reflects the subjectivity or bias involved in the landmark selection process.

On the analytical side a number of different methods of analysing these cephalograms has emerged in recent years as alternatives to what is now known as conventional cephalometric analysis (CCA). Examples of the latter are the Tensor Analysis (TA), Biorthogonal Grids (BOG), Finite Element Analysis (FEA), Elliptical Fourier Analysis (EFA) and Euclidean Distance Matrix Analysis (EDMA). In order to decide which available method of analysis would offer the optimum approach in the context of this particular project these different methods were compared.

1.3.2.2 Other cephalometric techniques

Alternatives to CCA have emerged, mainly as a result of the desire to improve the ability to measure shape or shape change. A number of these methods have been adopted by orthodontists from various biomechanical fields.

I) Tensor analysis (TA) was developed by Bookstein to deal with many of the shortcomings with conventional cephalometric analysis (Bookstein 1974, 1978, 1982, 1983, 1984) for the evaluation of shape changes. TA is based on the homologous point representation, an aspect it shares with CCA, but otherwise embodies an entirely different approach to the analysis of complex two dimensional forms.

By generating a series of triangles using homologous landmarks across the morphological form, one can compute the shape changes. These shape changes are computed as pairwise comparisons between the base triangle and its deformed pair. These triangles are independent of each other and the technique enables a comparison of form before and after deformation which is entirely independent of the co-ordinate systems or any local frame of reference. Bookstein (1978) emphasised that:

i) this method does not measure shape, it measures "shape change".

ii) this shape change refers to the change in shape of the triangles being used to represent the form, since the morphology is being reduced to sets of triangles.

iii) this proposed analysis for a single pair of triangles is fully equivalent to the simplest case of the finite element description with principle strains at exactly 90 degrees.

As a descriptive measure, the TA method is a novel way of looking at craniofacial data. It is co-ordinate free and provides summary estimates of internal shape changes not obtainable by conventional cephalometric analysis.

II) Finite element analysis (FEA) works on the principle that when a structure is loaded stresses are set up which tend to deform the material. This deformation leads to displacements in the dimensions of the structure and its application to craniofacial morphology presents deformation as a change in morphological shape (Huiskes and Chao, 1983).

The FEA represents an approach that is very similar to the tensor analysis method. It is again limited to homologous landmarks, and is invariant with respect to the co-ordinate system. It shares with the TA the concept of deformation and the "dilations" in the TA are called "strain" measures of a rigid body in an engineering sense. It differs from the TA in that it can be extended to three dimensions. By replacing triangles with cubes and using an initial form as a base, each element in the structure is pairwise compared, and the shape changes computed as a deformation. For each cube, or element, it is possible to estimate

shape and size differences and therefore form difference. FEA represents one of the few approaches that allows the description of three dimensional data, enabling analysis of growth behaviour in all directions. It has been applied to measurement of the cranium of an idealised non- human primate by Cheverud and Richtsmeier (1986) to analyse sexual dimorphism in facial growth in Rhesus macaque.

III The Elliptical Fourier Analysis (EFA) technique represents a different approach to circumvent the insufficiencies presented with conventional cephalometric analysis. It is based on a widely utilised technique employed primarily in engineering and physics for characterisation of wave forms. More recently the method has been increasingly applied in archaeology, biomechanics and the biological sciences (Healy-Williams and Williams, 1981; Rohlf and Ehrlich, 1982; Lestrel and Siriani, 1982; Gero and Mazzullo, 1984 and Lestrel, 1974, 1980, 1982 and 1989).

This technique of curve fitting represents a departure from conventional cephalometric analysis as well as both TA and FEA in that it is not dependent on homologous landmarks, although it can contain that information. Also in contrast to TA and FEA,the elliptical Fourier analysis is primarily intended for a description of outline or boundary information which is largely missing from the other two approaches and is even applicable to morphologies that have no clearly discernible landmarks, for example the cranial vault. A drawback however is that the elliptical Fourier analysis is currently limited to two dimensions. A study by Lestrel and Roche (1976) describes the application of the elliptic Fourier analysis. They compared 80 Downs syndrome individuals with 80 normal controls to try to ascertain whether cranial thickness in the mid-sagittal plane differs between the two groups.

IV) Euclidean Distance Matrix Analysis (EDMA) was introduced by Lele in 1990 as a method for quantitative comparison of the shapes of biological objects. The method can be used to describe the shape of anything that has recognisable landmarks. Three dimensional co-ordinate data are used to generate a distance matrix consisting of all possible linear distances between landmark pairs in the forms being compared. The Euclidean Distance Matrices are then compared by

calculating the ratio of all distances in the initial morphology to the same distances in a comparison object. This matrix of ratios is called the form difference matrix.

The form difference matrix allows determination of the way the two shapes differ by identifying those linear differences that are most and least different between the shapes being compared. EDMA therefore provides a means for identifying local areas of form difference as shown by Richtsmeier et al 1990). It also provides means by which size and shape difference in biological forms can be evaluated numerically and therefore handled statistically. EDMA has already been applied to the study of craniofacial morphology using standard cephalometric landmarks (Corner and Richtsmeier, 1991; Lele, 1991; Lele and Richtsmeier, 1991, 1992) and also to the study of dental arch shape and asymmetry (Ferrario *et al.*, 1993). Moreover unlike the elliptical Fourier analysis, EDMA can be applied to both two and three dimensional data.

1.3.2.3 Rationale for conventional cephalometric analysis

All of the above methods used for description of morphology and shape change do have merits and definite indications for particular applications in data analysis. None however can claim universal acceptance or to be the best method to apply in every circumstance. The following describes some of the limitations and impracticalities of these various methods and outlines the reasons for using conventional cephalometric analysis for the present study.

a) Landmarks

Landmark identification errors are a recognised limitation of the interpretation of cephalometric data. (see section 1.3.4.3) All of the above methods however, with the exception of the Fourier Analysis use landmark data, and would therefore be subject to the same degree of landmark identification error as conventional cephalometric analysis.

b) Orientation

Location of cephalometric outline points according to the maximum convexity or concavity of the outline of the structure will vary if the true vertical or

horizontal reference plane is used. Use of an anatomic reference line however will enable accurate reproducibility of such point identification irrespective of subject orientation. In the present study point definitions were such that they were not dependent on subject orientation in space.

c) Outline Shape

CCA tends to use a series of discreet points on an outline joined together by straight lines and in practice it is convenient to use anatomic points to a large extent with fairly wide spacings. This limits interpretation to comparison with similar data or with regional mean values. The use of the PC DIG programme overcomes this problem to some extent. The programme is designed to describe a curvature rather than a straight line between successive points and a greater number of points are digitised on outlines, where the rate of change of curvature is greater. For example, to describe the curvature of the pituitary fossa, points at much closer intervals will be required than for those defining the external cranial outline. Careful attention to the distribution of outline points using the PC DIG programme enables production of an accurate outline and therefore of accurate area measurements.

The elliptical Fourier method enables curved and irregular shapes to be not only plotted, but also analysed. There is no doubt that this represents a numerical method that can accurately characterise the shape of complex biological shapes which can be meaningfully compared. It does suffer from the drawbacks of cost in terms of time and resources given the large number of data points and an extensive number of harmonics that need to be computed to effect an acceptable fit. For example, a system of three hundred and thirty points and one hundred harmonics were used to yield a Fourier description of the complete craniofacial complex (Lestrel, 1989).

Another drawback is the difficulty in relating the values of the coefficients to the shape of various regions of an anatomical outline. This problem is related to the global effect each Fourier wave form has on an outline.

The ideal analysis of outline form would utilise landmark and outline data simultaneously, the advantage of homologous landmarks being the maintenance of the relative position of all biological loci of interest (Lele and Richtsmeier, 1991). It is apparent that no single method to date encompasses both the homologous point information (landmarks) and the boundary curve information (outline) into a single numerical model.

d) Internal Shape Changes

EDMA and FEA are methods used for the description of internal shape changes in three dimensions. Studies of shape change or growth change that use three dimensional biological landmark data maintain the geometric and biological integrity of an object and therefore can provide a comprehensive analysis of form change (Bookstein *et al.*, 1987; Cheverud and Richtsmeier, 1986).

In the present study such technology was not applicable for two reasons. Firstly, three dimensional data was not available for either the study or the control group, and secondly, for a cross-sectional comparative study, analysis of internal shape change on a longitudinal basis was not required.

In addition there are some practical problems associated with the FEA, such as complex statistical manipulation and interpretation of results because of the large number of three dimensional finite elements used for the description. CCA is an easier method to understand and apply, but it is unable to assess internal rotational changes that are demonstrable with the FEA technique.

e) Superimposition

To measure changes using conventional cephalometric analysis, it is necessary to superimpose X-rays or tracings. This requires a common reference plane such as S-N, the Frankfort plane, or some other plane for registration. Location of these planes is subject to error which can affect the accuracy of superimposition studies. EDMA, FEA and EFA, all eliminate superimposition problems, as the description of shape change does not depend on any local frame of reference or orientation plane.

In the present study the main emphasis is on statistical comparison of craniofacial parameters in two groups of subjects. Superimposition for the purposes of analysing longitudinal shape change or growth change was not required.

The objectives of the present study were limited to the use of lateral cephalometry (a) to analyse an appropriate sample of individuals for the preparation of normative cephalometric mean values and (b) to compare the size and/or shape of selected craniofacial elements of an individual with those of the normative standard.

It was mainly for the reasons of ease of interpretation of and statistical management facilitated by banks of existing data for comparison, that justified the use of the conventional cephalometric procedure. It was also considered that all the alternative methods described above do represent major improvements over CCA as either outline or shape change descriptors, but they must be considered as promising preliminary numerical models rather than final definitive pronouncements. Each method has constraints that precluded it from being the method of choice. It is interesting to note that a recent paper by Trotman and Ross (1993) compared the use of CCA and TA (the Biorthogonal Grid method). The results of the two methods of analysis were in agreement and tended to complement each other. With all factors considered the use of conventional cephalometric analysis was thought to be entirely appropriate in the context of this particular investigation.

1.3.3 Rationale for use of lateral cephalograms

The decision to use lateral cephalograms for the analysis of craniofacial and dental morphology was made for the following reasons -

a) Desire to carry out a meaningful analysis of the craniofacial and dental morphology in the parents of children with cleft lip and/or palate using the lateral cephalograms available.

b) Availability of suitable control material to match the lateral cephalograms recorded for the parental sample.

Previous studies have indicated that differences exist not only in the antero-posterior and vertical, but also in the transverse dimension when comparing parents of cleft children with a control. It would seem sensible to record postero-anterior cephalograms in a study such as this. Meaningful comparison, however, depends upon the availability of a suitable control. Postero-anterior cephalogram control data for a West of Scotland population does not exist, and it was not ethically justifiable to subject a random sample of the population to non-therapeutic X-rays for this purpose.

c) Of the studies in the literature which have examined craniofacial morphology, lateral cephalograms have been the most commonly used. This means that similar landmarks and parameters can be incorporated in this study which enables meaningful comparison with previous work done in the field.

1.3.4 Errors in Cephalometry

1.3.4.1 Subject positioning

When cephalometrics was first introduced to the Orthodontic profession in 1931, the feature that distinguished this technique from the taking of ordinary lateral skull radiographs was the "standardisation" of head positioning in a cephalostat.

It is possible to install the cephalostat machine with a fixed and reproducible relationship between the X-ray source, head holding apparatus and film. It is also possible to accurately measure individual differences that may exist between different patients (e.g. mid-sagittal plane to film distance) and theoretically measurements taken using the same techniques in different locations can be compared. It is not easy however to ensure that the subject head will be completely immobilised or that the head will be in exactly the same position between exposures. The ear posts are in a mobile cartilagenous canal and the nasion rest is placed on freely moveable tissue. As a result there is always enough freedom of movement to blur a film, and unlike a photographic exposure which has a very short exposure time (0.04 seconds), there is sufficient time for movement to blur the radiographic film during an X-ray exposure which takes 0.4-0.6 seconds.

Even if a subject does not move his entire head, he can still breathe, talk, smile, move lips and tongue, or even the entire mandible. Blurring due to motion can vary from being negligible to making a film worthless. It is therefore important that steps be taken to minimise inaccuracy due to subject motion during exposure.

1.3.4.2 Projection errors

a) Enlargement

A cephalogram is created by projecting X-rays from what may be considered a point source through the object some distance away and recorded on a film beyond the object. As such the X-rays are constantly diverging and so produce an enlarged image of the object on the film. Distances are the key to the degree of enlargement as;

$$\text{The percentage enlargement} = \frac{\text{distance from source to mid saggital plane}}{\text{distance from source to film}} \times 100$$

The magnification factor can be calculated provided these distances have been measured and are known. Correction for enlargement is simple if the same subject to film distance is used as the same correction factor can then be used. If different subject to film distances are used this must be recorded for each film.

In the cephalometric technique the film and the mid-sagittal plane of the patient are taken as parallel. This means that angular and ratio measurements in subsequent cephalometric analysis are unaffected by the enlargement factor, but correction of measurements is necessary for obtaining absolute linear values or for superimposing serial tracings, assuming the magnification to differ from registration to registration.

b) Distortion

Distortion is the result of the fact that the object in cephalometric analysis is three dimensional and those points and structures which are not on the mid-sagittal plane and outside the principal axis (central ray) are enlarged by varying amounts. Distortion is more serious than simple enlargement since it affects all parameters on a cephalometric film, angular, linear and proportional measurements. This is

further compounded if the true anatomic mid-sagittal plane of the head does not coincide with the normal mid-sagittal plane of the X-ray cephalostat system.

Many cephalometric points and distances are thus affected by distortion and one of the problems is the choice of a single point to represent bilateral structures. Right and left sides of the head will obviously differ in their distance from the X-ray source resulting in a difference in magnification between right and left sides and consequently a double image on the two dimensional cephalogram. Cephalometric analysis makes use of many such points including orbitale, first molars, the mandibular border, pterygo maxillary fissure and key ridge of the maxillary bone.

In traditional anthropometric cephalometry the convention with bilateral points was to use only the left side points for measurement. In orthodontic cephalometrics, this can introduce considerable error and by convention the problem is overcome by using the mid-point between bilateral structures. The points therefore chosen to represent these structures are projected onto the mid-sagittal plane and all measurements are made from this common base.

c) Blurring

Apart from the motion of the subject, film blurring may be a result of optical blurring or graininess of the film. In cephalometry, the X-ray source is not in practice a point source but a beam collimated by a rectangular collimator casting a rectangular shadow on the film. This shadow is not sharply defined at the edges, but has a fuzzy border known in optics as the penumbra. The X-ray image of an object projected on to the screen will also be surrounded by this penumbra, the width of which is directly proportional to the size of the X-ray target (source of the X-rays), and the distance of the object from the film. It is inversely proportional to the distance of the X-ray tube from the subject. To reduce optical blurring in cephalometrics, then, the objective is to get the patient as close as possible to the film, as far as possible from the X-ray source and to use the smallest possible X-ray target.

Broadway *et al.*, 1962 in an investigation of the accuracy of laterals cephalograms, concluded that to achieve the necessary high quality radiographic film it was desirable to:

increase the focus film distance to nine feet, decrease the distance between the sagittal plane and the film, and decrease the voltage (kV). These factors did improve the film contrast and the improvement in landmark clarity facilitated identification. Nevertheless these factors result in an unacceptable increase in the radiation dosage and the scattered radiation.

The five feet distance traditionally used in cephalometrics is a good compromise considering all the factors dependent on this distance and the film is placed as close as possible to the subject. Choice of X-ray tube with the minimum possible target size would therefore be the operators only control over the optical blurring. Unfortunately, however, a small target cannot handle as much current (mA) as a larger one, so what would be gained in reducing target size would be lost in prolonged exposure time with a greater risk of subject motion. Apart from optical blurring a further source of image blurring is the intensifying screen. The image intensification action of these screens is due to the fact that they contain mineral particles which glow on exposure to X-rays. The cephalometric image is a composite of all these glowing particles, each of which produces a small blurred spot on the film. Since this blurring is caused by the scattering of light, the extent of the blurred area depends on the amount of light being scattered. The edges of a very dark (radiolucent) area on the film produced by very brightly glowing particles will be more noticeably blurred than will be edges of a lighter (radiopaque) area.

Despite the above problems which results in blurring of the edges of the image intensifying screens are necessary for the technique for the dual purpose of reducing exposure time and reducing the intensity of X-ray dosage. In a report by Thurrow (1951) on the problem of blurring in cephalometrics, he believed that overall the effect of blurring on the accuracy of cephalometric measurement is such that it would be difficult to justify the expression of the dimension in units smaller than 0.5 mm.

Studies by Brodie (1949), Bjork and Solow (1962) and Salzmann (1964) pointed out the problems of projection errors in cephalometrics. Adams (1940), Wylie and Elsassar (1948), Vogel (1967) also recognised problems with projection errors and attempted to introduce correction factors. However the cumbersome nature of the

necessary computations has prevented the general use of these adjustments. Systematic correction for projection errors have been obtained either by the use of stereo head films or by the integration of information from lateral or frontal films. Neither of these methods is considered practical for routine clinical use (Baumrind and Frantz 1971a). It would seem that although complete control of projection errors is not possible their recognition and the recommendations discussed above will reduce them to an acceptable level. Also the fact that the study group and control cephalograms in this study have been taken with similar apparatus, the magnification factor for which is known, means that valid comparisons can be made.

1.3.4.3 Landmark identification errors

Many investigators have shown that inconsistency in identification of cephalometric landmarks is an important source of error in cephalometry (Bjork, 1947; Hixon, 1956; Hatton and Granger, 1958; Savara *et al.*, 1966; Richardson, 1966; Baumrind and Frantz, 1971a). These errors tend to be specific to each landmark and some studies, e.g. Richardson (1966) have attempted to rank the reproducibility of points by looking at the means and standard deviations of repeated measurements.

Richardson (1966) carried out an investigation into the reproducibility of some points, planes and lines using cephalometric analysis. Ten lateral cephalograms were traced by two observers on two different occasions and superimposition on De Coster's line was used to determine inter- and intra- observer discrepancy. The discrepancy in point identification in both the horizontal and vertical planes was determined: each mean difference being tested against the theoretical expectation of low discrepancy (under the null hypothesis) using the student "t" test. The difference between inter- and intra-observer accuracy and between vertical and horizontal reproducibility for each point was highlighted. It was concluded that the discrepancies between measurements made by different observers were more serious than those made by the same observer on different occasions.

Each point had a characteristic elliptical envelope of error due to varying vertical and horizontal components. For example nasion (N) ranked higher in the order of

reproducibility horizontally, than vertically, whereas menton (Me) was much more reproducible vertically than horizontally and orbitale (Or) showed a relatively low level of reproducibility in both dimensions. Anterior nasal spine (ANS) and posterior nasal spine (PNS) were much more reproducible vertically than horizontally. This has definite implications for the choice of parameters used in the cephalometric analysis. The author concluded that with regard to angular measurements the maxillary, mandibular and sella/nasion planes were the most accurate since PNS, ANS, gonion, menton, sella and nasion showed acceptably small vertical discrepancies. The Frankfort plane, on the other hand was not recommended because of the vertical and horizontal variation of its defining points, orbitale (Or) and porion (Po). The deficiencies in this paper were the fact that only ten radiographs were used and there was no report of a time interval between successive tracings by each observer. Also it would have been interesting to analyse the combined variation for both the vertical and horizontal axes for each point using a multivariate statistical analysis or Hoteling's t-test.

Baumrind and Frantz (1971a) conducted a study on the reliability of landmark identification using 15 well known cephalometric landmarks on a random sample of 20 lateral cephalograms. Each of the 20 films was traced by five members of a graduate class who had just completed a training course in cephalometric diagnostics. They had therefore received the same information on landmark definition. As in the Richardson study the magnitude of error in landmark identification was determined in both the x- and y- axis. They found that the distribution of error was systematic and each landmark tended to have a characteristic distribution of error. He reported that (a) the sharpness of the edge affected the point to be identified, (b) the superimposition of adjacent structures because of the two-dimensional image and (c) the individual interpretation of the definition of the landmarks were the three major factors which infringed on the accuracy of landmark identification. The observation that lower incisor apex (Ali) and gonion (Go) were the least reliable landmarks and sella (S), despite the fact that it is a visually interpreted point was one of the most reproducible. The recommendation from this study was that the impact of the observed errors in landmark location can be reduced

by taking the average value of repeated (same operator) or replicated (different operators) estimates of landmark identification.

Midtgard *et al.* (1974) also investigated reproducibility of cephalometric landmarks and measurements. The study involved taking two separate cephalograms of each of the sample of 25 randomly chosen children. For each case the lateral cephalograms were superimposed on the bony detail of the anterior and posterior cranial bases. They studied 15 cephalometric landmarks and seven linear measurements. In the first part of the study the same observer recorded the landmarks on the two consecutively taken radiographs of the same individual child. In the second part each cephalogram was traced twice by each of two observers with an interval of one month between tracings.

The statistical methods used were the student "t" test and the method error was examined using the ratio of the error variance from measurements of the same films to the between- subject variance. It was found that the placing of different landmarks on the two consecutively taken lateral cephalograms of the same child showed varying degrees of uncertainty. The degree of uncertainty varied from landmark to landmark with the greatest difference between the two recordings being for orbitale (Or) where the mean difference was greater than two millimetres. The most reliable landmarks were sella and articulare. The positions of the landmarks on the same lateral cephalogram on two separate occasions by the same observer with an interval of one month showed approximately the same degree of variation as the inter-observer differences when tracing the consecutively taken cephalograms of the same individual. The authors concluded that -

i) on two consecutively taken radiographs of the same individual, there was a similar range of error in identification of the landmarks used in this analysis.

ii) The interval of one month between repeat tracings of the same cephalogram by the same operator did not significantly affect reliability of landmark identification.

iii) There were no significant differences in landmark identification between two different observers on the same film.

iv) The main source of error in cephalometric measurements would seem to be due to the uncertainty of the observer in placing the landmarks.

v) With calculation of cranial distances, the greatest uncertainty was found in the calculation of distances nasion to A-point and nasion to B-point.

Broch *et al.* (1981) used direct digitisation by one observer in thirty randomly chosen lateral cephalograms to study in landmark identification. Fifteen commonly used landmarks were digitised twice, the recordings carried out one month apart, and the method error calculated for each landmark. Each landmark had its own characteristic envelope of error along the x- and y- axis, similar to the findings of Richardson (1966) and Baumrind and Frantz (1971a). The authors concluded that with care it is possible to achieve good reproduction of most landmarks. Some, however, are not easy to locate reliably, for example basion (Ba), anterior nasal spine (ANS) and mesio-buccal cusp of upper first permanent molar (UMT). The consequences of this are much more significant in a single case or when measuring small dimensions.

A further study on the inter- and intra- observer reproducibility of cephalometric landmarks was carried out by Stabrun and Danielsen (1982). Fifteen landmarks were registered using a digitiser and the two observers repeated the registration of landmarks one month later. For each registration the observers were also asked to report the certainty or uncertainty with which they felt they had located the lower incisor apex; the rationale of this being that previous studies have shown lower incisor apex to be difficult to identify. The difference between the means on each occasion for each observer were compared by two tailed "t" tests. As in previous studies the results showed that each landmark had a different degree of reproducibility. Points A (subspinale) and B (supramentale) had a wide distribution along the y axis and those for anterior nasal spine (spinale) showed a wide distribution along the x axis. The intra-observer data indicated that each observer held a definite opinion regarding the landmark definition which resulted in improvement in individual precision. Inter-observer differences on the other hand showed significant disagreement as to the application of the definition of some landmarks despite prior calibration training. Finally the authors reported that location of

the lower incisor apex was uncertain in 75% of cases and this point does not have a high degree of reproducibility.

The optimum method therefore for precision of landmark identification is for the same observer to measure each cephalogram twice with at least two week interval between recordings. Precise definition of landmarks and rejection of poor quality films are also important. The selection of landmarks to be used in a study and the interpretation of the results should be determined by the landmark reproducibility.

1.3.4.4 Measurement errors in cephalometry

Cephalometric analysis has traditionally been carried out on a tracing of the radiograph which is then measured using a ruler and protractor. More recently the use of electronic digitising apparatus has allowed mathematical calculation of angles and distances using Cartesian (x and y) co-ordinates from the digitisation of landmarks. These can be obtained either directly from the radiograph, or from a tracing of the radiograph (Richardson, 1981; Houston, 1982). The use of video imaging techniques in which a cephalometric radiograph is captured using a video camera, the image being stored digitally has been reported by Jackson *et al.* (1985). This technique allows processing of the captured image to enhance areas of interest . Another system of digital storage and manipulation of the captured video image has been described by Oliver (1991). This system, known as ISI, comprises a video camera mounted in a light box and on line to a high resolution image monitor and computer. This system aims to enhance the video image of the radiograph prior to tracing or landmark identification. It is important that having collected cephalometric data an accurate and reproducible method of measurement is used in its analysis.

The use of a digitiser to record the co-ordinates of each landmark has advantages in terms of speed in the analysis of data as an adjoining computer can be programmed to calculate linear, angular and area parameters chosen by the operator. The system can also be used to compute data from groups of subjects, this facility being especially useful when large numbers of records are to be analysed. The accuracy of the digitiser and its influence on the error of measurement of a line of a given length depends on the

resolution of the digitiser and reliability with which the end points of the line can be identified (Houston, 1979). Resolution is defined as the shortest distance which can be distinguished between two points.

The GTCO Digitiser used in this study was tested for resolution and was found to have an error of no more than 0.11 mm for the y co-ordinates and 0.18 mm for the x co-ordinates. Digitisation of the radiograph was preceded by digitising four Fiducial or Cartographic points in order to create a co-ordinate system specific for that image. These must be placed in precisely the same position on the radiograph prior to each repeat digitisation and pin point markings on duplicate films were used. In this way it is possible to return to the image at a later date and by redigitising the Fiducial points recreate the same co-ordinate system, thereby enabling the user to re-register points or add new landmarks to those already recorded. In the absence of Fiducial points inclusion of new landmarks entails redigitising of the entire image.

Since the introduction of computerised direct digitising technology, a number of studies have addressed the question of its accuracy compared to traditional manual methods of cephalometric analysis. Gravely and Benzies (1974) investigated the clinical significance of tracing error in cephalometry. They reported that measurement errors associated with tracing of cephalograms include the perceptive limits of the human eye and thickness of the pencil line. Their study involved 103 cephalograms traced by three operators under optimum conditions. Each film was traced on two separate occasions by each of the three tracers. The standard deviation between the first and second measurements was calculated and the confidence limits for all measurements for the three tracers in the study. The results of this study showed that cephalometric tracing errors are high, even when tracings are done by experienced orthodontists. The authors' conclusions were that:

i) tracing errors are mainly due to landmark identification;

ii) tracing should be repeated to minimise error;

iii) a tracer should establish his own method error as part of the study before data can have meaningful interpretation.

Richardson (1981) investigated the question of the relative reliability of tracing and direct digitisation. In his study repeated digitisations were made for fifty lateral skull radiographs. Following this the points were marked on acetate tracing sheets and their co-ordinates were measured to the nearest 0.25 mm using a ruler. Each of the two methods was repeated on separate occasions and for both methods the discrepancy between the first and second measurements of each point was calculated. Means and standard deviations of the discrepancies between occasions were derived and compared. The results were presented in separate tables to compare the reproducibility of each point in both the horizontal (x) and vertical (y) components of the co-ordinates.

For 12 of the 14 points the mean horizontal digitisation discrepancy was smaller than for the traditional method and when comparing standard deviations digitisation was superior for nine of the 14 points. In the vertical direction the mean discrepancy of the digitiser measurements was smaller than the traditional method for 12 of the 14 points and the standard deviation of the digitiser measurement was less than the corresponding manual measurement for eight of the 14 points. The authors suggested that the digitiser could be used to find and record points in a curved outline by running the digitiser cross wires across the curves in a horizontal or vertical direction. This enables the operator to identify horizontal and vertical concavities of convexities provided the film is at the correct orientation. His results also showed that the traditional methods, although inferior were only marginally so and points such as the apex of the lower incisor and anterior nasal spine seemed to be more accurately reproduced using the traditional methods.

Houston (1982) also compared the reliability of measurement of cephalometric radiographs by tracings and direct digitisation. He identified 13 points on each of twenty five lateral skull radiographs. These were traced on two occasions a week apart and on each occasion the tracings were digitised twice. In order to avoid the risk of memory affecting the results, the second digitisation of the radiographs was delayed for six months; and as with the tracings, the digitisation was repeated one week later. Fiducial points were used so that the images could be superimposed to allow calculation of the differences in landmark co-ordinates. The first and second tracings were compared as

were the repeated digitisations. The evaluation of error in landmark identification was slightly different. For the tracings the differences between the repeat measurements for linear and angular measurements were calculated and for the repeated digitisation, the distance between repeat co-ordinates was calculated, the images being superimposed on the Fiducial points. The results of this study showed that the errors in direct digitisation on two separate occasions were greater than those of repeat tracings. The author explained that the tracing of an indistinct structure, such as the apical region of the lower incisor aids in the location of the apex point. He also pointed out that the design of the cursor used for digitising tended to obscure the structures peripheral to the landmark of interest and sometimes the cross hairs of the cursor were not easy to see against darker parts of the radiographic image, problems that do not arise with the digitisation of tracings. It is also worth noting that as in the Richardson study, the actual differences between measurements made using the two methods were only marginal.

Cohen (1984) evaluated the use of the digitiser on tracings, untraced films and modified "dot" tracings. He found that the errors in measurement of conventional cephalometric angles were comparable irrespective of the method chosen, but errors in landmark location were smallest when measurements were made directly on his 20 lateral skull radiographs. The reasons suggested was that two guesses have to be made when measuring and tracing; firstly in drawing the tracing of the radiographic landmark, and secondly, in attempting to record the exact position of the pencil line which has a significant width. He felt that the more direct the observation, the less room there was for error.

Sandler (1988) also carried out a cephalometric study to compare and contrast the errors involved in taking linear and angular measurements using three different methods. These were using the traditional hand instruments for tracing, digitisation of tracings and direct digitisation of the radiographs. Twenty five radiographs were selected according to the quality and provided there were no partially erupted or unerupted teeth which would make the lower incisor apex identification difficult. Each radiograph was traced on four occasions, twice to allow manual measurements to be carried out and twice to allow digitisation from the tracing. There was at least one week between tracings of the

same radiograph to avoid risk of memorisation of landmarks. For the manual tracings, angular measurements were estimated to the nearest 0.25° and linear measurements to the nearest 0.25 mm. Results were analysed using the standard deviations of the differences between replicate measurements. A one sample "t" test was also conducted to detect systematic error within the methods used. For each variable with each method, the absolute errors represented by the standard errors for a single determination were calculated by Dalberg's method.

The results of this study showed that the errors in the traditional manual method were slightly less than those using digitised tracings, but slightly greater than those when digitising directly. The differences between the methods were not marked, and as in previous studies, certain manual measurements were more reproducible than either of the digitising methods. Direct digitisation showed higher standard deviations than both the other methods with measurements involving cephalometric points gonion and articulare. The author commented that points such as those which are not on the mid-sagittal plane tend to give a double image and location of these points is more accurate if they are traced prior to digitising. This study did not show the higher standard deviations for many points using direct digitisation that were reported by Richardson, 1981 and Houston, 1982. This is however, not surprising, since the radiographs were selected with clarity of lower incisor apex as one of the criteria and the one week time interval between repeat tracings may not have completely eliminated memory bias. Among the author's conclusions were that:

i) there is an appreciable amount of error in taking cephalometric measurements from radiographs whichever method is chosen.

ii) angular measurements involving the incisor teeth had consistently high error and therefore these must be treated with caution.

iii) manual measurements, if done carefully, compare well with methods involving the digitiser and there is no reason why results using traditional methods should be considered any less valid.

Oliver (1991) compared five different methods of cephalometric analysis by repeated analysis of the same five lateral skull cephalograms. A simple 12 point analysis was carried out using the following five methods -

i) Manual tracing

ii) Digitisation of the manual tracing

iii) Direct digitisation of the radiograph

iv) Digitising an enhanced video image of the radiograph, and

v) Using the image enhancement apparatus with the tracing.

Results were presented with the standard deviation estimates for each method of measurement for each variable studied. The findings of the study were that direct digitisation of lateral cephalograms is less reproducible than either the traditional method of measurement or digitisation of the tracing. Also there did not appear to be any improvement in reproducibility using the image enhancement techniques.

The standard deviation for SNA, ANB, MMPA and upper incisor angulation was shown to be approximately twice as great for direct digitisation compared with digitisation of the tracing. Using the standard method of direct digitisation, there was little difference between the values for digitisation of the tracing and direct digitisation, but neither method was quite as reproducible as the manual method of tracing. The image enhancement techniques were however consistently less reproducible and there were slightly larger standard deviations for the direct digitisation than with the tracing digitisation using the image enhancement technique. This means that of all the methods used the direct digitisation using image enhancement techniques showed the poorest

measurement reproducibility. The author suggested that a possible explanation for the twofold increase in standard deviation for SNA and ANB when comparing direct digitisation of the tracing was the error in identification of A point. The envelope of error for A-point in this study has more horizontal distribution than was found by Richardson (1966) and Baumrind and Frantz (1971a). The author concluded that for everyday clinical usage, it is doubtful that the levels of difference between the methods of measurement have any great significance, although it would be desirable to obtain higher levels of reproducibility if the image enhancement techniques are to be used for precise research purposes.

The contrasting findings of this study in terms of the superiority of the manual method with those of the previous study (Sandler, 1988) may well be explained by the quality of films used; those for the Oliver study (1991) were chosen at random while Sandler (1988) used only high quality films. There is no doubt that instant identification of anatomical landmarks is much easier in the better contrast films and a tracing provides more time and the opportunity to apply some expert knowledge in those cases where points are more obscure. These observations would be in agreement to those of Richardson (1981) who felt that the greatest advantage of the digitiser was when measuring reliably reproducible cephalometric points. A tracing will undoubtedly have positive advantages where there are double images; and in an analysis where constructed points are used an intervening tracing is indispensable.

1.4 HEREDITY AND CRANIOFACIAL MORPHOLOGY

1.4.1 Historical background

Review of the orthodontic literature over the last 50 years reveals a consistent interest in the inheritance of craniofacial morphology.

Iwagaki (1938) is usually credited with the first attempt to study the heredity of malocclusion. He analysed over two thousand Japanese family pedigrees to ascertain the influence of genetics on mandibular prognathism. Rubbrect (1939) also studied prognathism and concluded that there was an irregularly dominant pattern of inheritance. These and other early genetic studies (Hughes and Moore, 1941; Curtner, 1953) did not use statistics to substantiate their findings and they interpreted their results in strictly Mendelian terms. Hughes and Moore (1941) concluded that craniofacial growth is under strong hereditary control and subscribed to a multiple gene concept of inheritance. They observed that mandibular and maxillary morphology is totally independent and furthermore in the mandible "the ramus, body, angle, alveolus and teeth are not too dependent on each other" since each feature of the craniofacial morphology is a multiple factor trait.

After the pioneer work of Broadbent (1931) many studies used cephalometric distances and angles to quantify craniofacial variation, one of the first of these being Wylie (1944) who studied one hundred pairs of twins using measurements similar to Broadbent's and he illustrated the effect of inheritance on variability of various craniofacial parameters. Stein *et al.* (1956) studied influence of heredity on malocclusion using angular cephalometric measurements only and concluded that there is a greater correlation between siblings than between parent-sibling combinations.

Watnick (1972) stated that a more quantifiable and more intricate method of analysis was required to study the heritability of craniofacial morphology and he devised a method of analysis using lateral and postero-anterior cephalograms and a template designed to divide the cephalometric contours into equal parts for digitisation. His method of statistical testing included multivariate and univariate analysis of variance of vector and area differences between curve pairs.

These and numerous other studies lead to the overall conclusion that craniofacial morphology in general is influenced by both genetic and environmental factors. The relative contribution of each varies from region to region in the orofacial, cranial and dental structures and from individual to individual.

1.4.2 Parental craniofacial morphology and cleft lip and palate

The fact that there is overwhelming evidence indicating a genetic contribution to craniofacial development has led to investigation into the relationship between parental craniofacial morphology and cleft lip and palate in siblings.

Carrick (1954) made one of the earliest references to the genetics of cleft lip and palate and his comprehensive literature review concluded that genetic studies demonstrated the probability of hereditary predisposition to clefting.

Trasler (1965, 1968) observed the relative susceptibility of two inbred strains of mice to cleft lip induced by maternal treatment with "aspirin" (acetylsalicylic acid) and he noted that differences in response seemed to depend on differences in the shape of the face.

In 1970 Fraser and Pashayan studied the facial morphology of parents of children with clefts of the lip and palate and found that compared to controls they had underdeveloped maxillae, wide bizygomatic diameters and thinner upper lips.

Coccaro *et al.* (1972) used lateral cephalographs to compare craniofacial parameters in parents with and without cleft lip and palate offspring and concluded that as well as having shorter vertical and horizontal measurements of the upper face and shorter nose length the mandible tends to be more prognathic.

These findings were supported by Shibasaki and Ohtsuka (1978) who also recorded the thin upper lips as indicated by Fraser and Pashayan in the parents of cleft children. They also found a significantly greater cranial base flexure angle in these parents as did Coccaro although Ross (1965) and Mars and Houston (1990) reported this angle to be normal in the children with clefts themselves.

Nakasima and Ichinose (1983, 1984) looked at facial morphology in the parents of children with cleft deformities but also examined the brain case and concluded that larger

horizontal and shorter vertical dimensions of the upper face and a significantly smaller brain case relative to controls seems to indicate a genetically determined morphology predisposing to the production of cleft lip and/or palate anomaly.

The authors of these cephalometric and anthropometric studies have attempted to identify individuals at greater risk for producing a child with a cleft. They generally imply that the deviations from "normal" or control craniofacial morphology may represent the extreme limit of the normal variability, and that the genes responsible for the deviation from normal contribute to the manifestation of clefting in their offspring. It is feasible, therefore that cephalometric studies could enable the identification of a phenotype which could be used to identify individuals who possess cleft lip and/or palate genotype.

Table 2 Previous cephalometric studies on parents of children with orofacial clefting.

	Author	Year	Race	CP	CL(P)	Females	Males	Total
1	Fraser and Pashayan	1970	Caucasian	-	50	25	25	50
2	Coccaro *et al.*	1972	Caucasian	-	40	20	20	40
3	Kurisu *et al.*	1974	Caucasian	124	223	206	141	347
4	Shibasaki and Ohtsuka	1978	Japanese	?	?	?	?	?
5	Nakasima and Ichinose	1983	Japanese	52	450	251	251	502
6	Prochazkova and Tolarova	1986	Czechslovak	40	-	20	20	40
7	Sato	1989	Japanese	28	172	100	100	200
8	Ward *et al.*	1989	Caucasian	-	82	?	?	82
9	Blanco *et al.*	1992	Chilean	-	22	15	7	22

Table 2 gives brief details of the composition of subjects in a number of previous cephalometric investigations into parental craniofacial morphology and section 4.1 includes a comparative analysis with the results of the present study. Agreement about which cephalometric variables most effectively characterise these parents is however lacking, making it impossible to draw firm conclusions regarding a direct association

between craniofacial morphology and cleft lip and palate. This may be explained in part by the diverse nature of the studies; there was no standardised protocol on subjects, methods, number involved and handling of the data making direct comparison inadequate. Ward *et al.* (1989) were also critical of the fact that previous studies tacitly accepted the multifactorial threshold model for the transmission of clefting deformity. They felt that the assumption that both parents contribute predisposing factors to an affected child could provide misleading results as it makes no allowance for the possibility that one parent may contribute more to the susceptibility for oral clefting than does the other. The emergence of evidence which suggests that one or more major genes may be responsible for clefting (Fogh-Anderson, 1942; Marazita *et al.*, 1984; Chung *et al.*, 1986; Eiberg *et al.*, 1987; and FitzPatrick and Farrall 1993) makes this a valid criticism and changes the interpretation of the data. The emphasis of future investigations should therefore be directed towards:-

i) identifying whether parents have distinct differences in their craniofacial morphology when compared to a control and if so how these differences are distributed between parental pairs.

ii) identifying whether there are genotypic differences between parents of cleft lip and palate probands and a control, by investigation of candidate gene allele frequencies.

Other questions which arise are;

i) is the difference in aetiology between CP and CL(P) reflected in the craniofacial characteristics as suggested by previous studies?

ii) are there any gender differences in the phenotype as some previous studies have suggested?

Clear definition of phenotypic form for CP and CL(P) would be very valuable in molecular linkage studies because of the ability to identify potential carriers of the gene or genes for clefting. By utilising recombinant DNA technology and linkage analysis the existence of genetic factors in the aetiopathogenesis of clefting may be identified.

1.5 AIMS OF THE PRESENT STUDY

The aim of this study was to try to identify parental characteristics (phenotypic or genotypic) which were associated with an increased risk of having a child with CL(P) or CP.

In order to carry out this dual phenotypic/genotypic study the plan of action was as follows:

i) To analyse lateral cephalograms of parents of a completely ascertained sample of children with CL(P) and with CP and compare with controls from the same West of Scotland population.

ii) To genotype parents of children with CL(P) and CP for restriction fragment length polymorphisms (RFLPs) at the TGFα locus and compare with a control population.

iii) To compare the TGFα genotype with observed cephalometric craniofacial variables in the parental sample.

CHAPTER 2

SUBJECTS AND METHODS

2.1 CHOICE OF SUBJECTS

2.1.1 Parental Sample

The study population comprised of those parents of infants born in the west of Scotland over a five year period from 1 January 1980 to 31 December 1984 (Table 3). Table 3 refers to the numbers of patients from each cleft category and further details of this completely ascertained sample have been reported in a recent publication (FitzPatrick *et al.*, 1994). The present study aimed to examine a sample of parents whose children had non-syndromic clefts and no other associated abnormalities. A careful history was taken to determine that the parents used were in fact the biological parents of the cleft proband. When the parents of those with chromosomal disorders, Stickler syndrome, Van der Woude syndrome, Pierre Robin syndrome, known teratogenic exposures (e.g. phenytoin or alcohol) or any other associated abnormality were excluded 152 parental "pairs" were left.

Table 3 Cleft types of probands in completely ascertained sample and subgroup who were involved in the present study.

		Type of Cleft			
Gender	Sample	(CL)	(CLP)	(CP)	Total
Male	I	31	59	66	156
	II	8	16	10	34
Female	I	20	29	81	130
	II	1	3	15	19
Total (%)	I	51 (17·8%)	88 (30·8%)	147 (51·4%)	286 (100%)
	II	9 (16.9%)	19 (35·9%)	25 (47·2%)	53 (100%)
TBP* ‰		0·27 ± 0·04	0·47 ± 0·05	0·79 ± 0·065	1·53 ± 0·018

I = completely ascertained sample*

II = probands whose parents participated in present study

TBP = total birth prevalence

2.1.2 Control Group

Following parental subject selection the matching with a carefully selected control group is one of the most critical aspects of a comparative study. In the present study the parental group was matched by a separate control group according to the availability of existing control material.

The taking of lateral cephalograms involves a degree of exposure to radiation which is cautiously controlled in contemporary medical practice. The taking of parental lateral skull radiographs in the context of this study was considered to be justified, but it would not be ethically permissible to do the same for a randomly chosen control population. The control material for this study was therefore taken from existing records in the archives of Glasgow Dental Hospital and School. This of course represented, not a random sample, but a referred sample of orthodontic patients. One major advantage of this approach is that both groups were derived from the same West of Scotland population.

Since the characteristics of the parental sample of subjects was known after their data was collected and analysed it was possible to select a control group with characteristics which matched as closely as possible. The criteria which were used in this selection process were as follows:

I) Gender: Roughly equal numbers of male and female subjects were required to match the parental sample. A number of one-parent families were however involved in the study with the mother being the participating parent in all but one such case. The control group consisted of 50 female subjects and 49 male subjects to reflect this.

II) Age: The mean age of the overall parental sample was 37 years and 2 months (Figure 10). The mean age of the mothers was 35 years and 8 months (SD 5·5 years and range, 24·5 - 48·8 years) and of the fathers was 38 years and 6 months (SD 5·1 years range 27·2 - 51·0 years). An adult orthodontic population tends to be somewhat younger than this and therefore the criterion used was that for the control group. The lateral cephalograms used were taken at the age of 18 years or older. The mean age of the control group was 22 years and 9 months, with the mean of the females 22·3 years (SD 3·2 years, range 18·4 - 34·1 years) and that of the males 23·3 years (SD 4·0 years, range 18·1 - 41·7 years). The amount of craniofacial growth between the age of 23 and 37 years would be expected to be negligible.

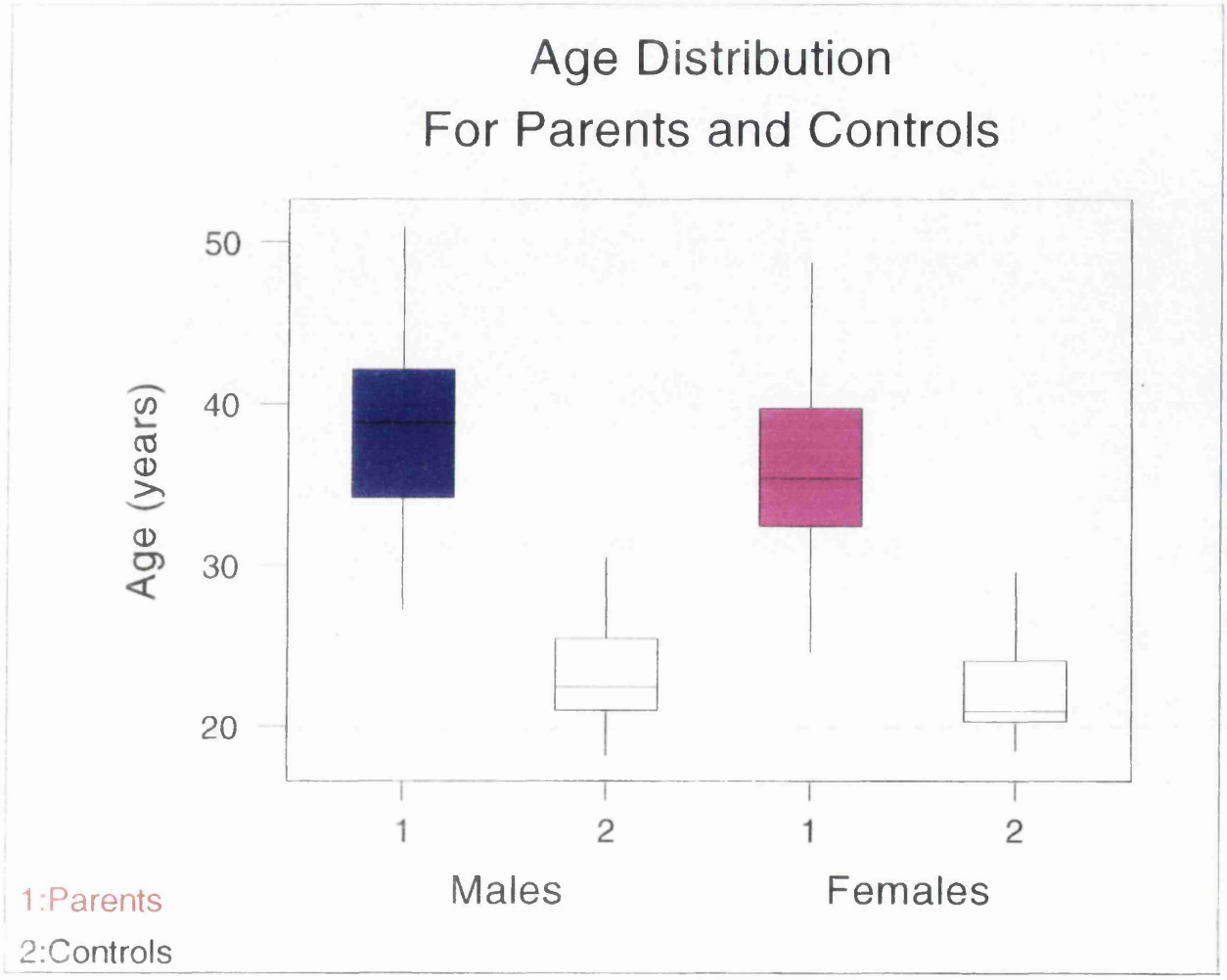


Figure 10 Age distribution for parental and control groups labelled by sex

III) Race: Since all the subjects in the parental group were indigenous to the population of the West of Scotland this made foreign nationals an obvious exclusion criterion when choosing the control. Although it was not possible to check the history of each of the control subjects their records indicated they were Caucasian and had a home address in Glasgow or the West of Scotland at the time of their attendance at Glasgow Dental Hospital and School.

IV) Malocclusion: Cephalometric studies such as this done in the past have tended to use cephalometric "norms" within their own population. Others have sought a selected sample of the population which demonstrates only normal skeletal and occlusal features. (Scheideman *et al.*, 1989)

These are considered acceptable as indices for comparison of an anomaly sample.

Analysis of the parental sample in this study reveals, however that there is a range of malocclusion types represented and a much more sensitive index for comparison is a control group which exhibits a similar range of malocclusion. Put another way, the comparison of a group of individuals of varying craniofacial morphology with a control which is specifically chosen as being normal is much more likely to throw up false positive differences than using a control from the same genetic pool which also contains a similar range of malocclusions. In the latter case any differences detected on analysis can more confidently be attributed to the difference in phenotype between the groups than to differences due to malocclusion type. Hence the use of this criterion in choosing the control group which was matched as far as possible for malocclusion.

I) Previous Dental History: Subjects who had previous fixed appliance orthodontic treatment or maxillofacial surgery were excluded. Two parents who had received orthodontic treatment in the form of a simple removable orthodontic appliance in the past were however retained. Subjects who were edentulous in one or both jaws were not excluded, nor were those who were partially edentulous and demonstrated an obvious degree of mandibular overclosure as a result. (However those of the parental sample who were either partially or totally edentulous in one

or both jaws were noted so that parameters which would be inaccurate as a result of an edentulous adjusted mandibular position were not measured.)

II) Record Quality: Radiographs were excluded if the entire skull and facial bones were not recorded on the film, or if due to exposure, developmental or other defects, the quality of the film was poor on subjective evaluation by the author. It is also critical that the magnification factor be known and for this reason all films taken outside Glasgow Dental Hospital and School were excluded from the study.

Five hundred and fifty two lateral skull radiographs of subjects of 18 years or older were available in the Glasgow Dental Hospital and School archives dating back to 1946. By subjective evaluation the cases were divided into mild, moderate and severe malocclusions. Moderate and severe Class II, Division 1, Class II, Division 2, Class III and all anterior open bite malocclusions were removed from the data, leaving 258 cases. These were then divided into the four recognised malocclusion categories (Class I, Class II, Division 1, Class II, Division 2, and Class III). These were further subdivided into male and female records giving 180 female and 78 male radiographs. From this "previewed" data, 50 male and 50 female radiographs were randomly chosen in proportion to the prevalence of malocclusion in the parental sample whose craniofacial morphology had already been analysed. Later tracing and analysis revealed the difference between the two groups on the basis of antero-posterior and vertical skeletal patterns (ANB and MMPA measurements respectively) after this visual subjective matching (Table 4).

Table 4 Distribution of malocclusion in parents and control classified according to skeletal class and MMPA.

	Skeletal Class			MMPA			
Subjects	I	II	III	High	Ave	Low	TOTAL
Parents	36	24	34	10	32	52	94
	(38·3%)	(25·5%)	(36·2%)	(10·6%)	(34·0%)	(55·4%)	(100%)
Control	45	29	25	5	40	54	99
	(45·5%)	(29·3%)	(25·2%)	(5·05%)	(40·4%)	(54·55%)	(100%)

2.2 DATA AND SAMPLES COLLECTED

There were two distinct episodes of data collection involved in the study, the taking of lateral cephalograms and the collection of a blood sample from each of the parents who volunteered. For rather unusual reasons these were not done on the same visit. Having been granted ethical approval for the taking of lateral cephalograms the subjects were appointed and on the same visit an exfoliative cytology sample of oral mucosal cells in the form of a mouthwash was collected. The latter is a non-invasive procedure for which ethical approval was not necessary and after centrifuge the pellet of exfoliative cytology cells obtained from each parent was stored at -70°C.

Later attempts to extract genomic DNA from these exfoliative cytology samples proved difficult and inconsistent results were being obtained. Blood samples are known to be a reliable source of DNA and a representative sample of the cleft palate probands as well as the parents were subsequently asked to participate in the donation of a blood sample for DNA analysis. Ethical approval for the same families as those who had participated in the cephalometric study was obtained and 10 ml blood samples from those who volunteered were collected.

2.2.1 Lateral Cephalograms

Having obtained ethical approval for the taking of cephalometric radiographs a letter was sent to the addresses of those 152 shortlisted families (see section 2.1.1) inviting volunteers to participate in the cephalometric study. It was necessary and appropriate that this letter mentioned the small risk that is thought to be associated with exposure to X-radiation. For various reasons such as moving from the address on our database, marriage breakdown, inconvenience due to job or transport and unwillingness to participate, this yielded 68 replies (44·7 %), and therefore a possible total of 136 parental subjects. A further drop out of 15 families during the data collection left a final

study group of 53 families willing and able to participate. Table 3 gives a breakdown of the types of clefts in the siblings of these parents and fortunately these are proportionately representative of the completely ascertained sample.

The overall sample of 106 parents who presented themselves for record collection are listed in Appendix A. Twenty three of these subjects were, however, found to be edentulous in one or both jaws and were therefore excluded. Table 5 gives the final composition of the parental sample for whom lateral cephalograms were taken.

Table 5 Composition of parental and control cephalometric and genetic study samples and number of individuals participating in the genetic study classified according to clefting status

	Cephalometric study			Genetic study		
Subjects	Parents		Control	Parents		Control
	CP	CL(P)		CP	CL(P)	
Female	17	25	50	18	21	62
Male	18	23	49	17	20	
Probands	0	0		8	11	
Totals (%)	35 (42·2%)	48 (57·8%)	99	43 (45·3%)	52 (54·7%)	62
All	83		99	95		62

All radiographs were taken using the same cephalometric equipment (Orthoceph 10, Siemens). The subjects were all positioned with the right side of their face oriented towards the X-ray tube. The optimum exposure was determined by a preliminary trial as 74 kV, 15 mA and 0.64 ms. The film (Kodak T Mat L) was exposed in cassettes equipped with intensifying screens (Kodak Lanex Medium) and no grid was used.

2.2.2 DNA Samples

A letter of explanation regarding the DNA analysis was sent to those 53 families of the completely ascertained sample who had volunteered to have cephalograms taken. They were offered the alternatives of having the blood samples taken either at the

Glasgow Dental Hospital or by their own General Medical Practitioner and the appropriate action was taken. This yielded a success rate of 74% with co-operation from 39 of the 53 families involved (see Table 5). It was suggested to the parents that a representative sample of cleft lip and palate children would enhance the study and 15 of the affected children volunteered to provide a blood sample. The 10 ml blood samples were delivered to the laboratory in anticoagulant (EDTA bottles) within 24 hours, and the DNA extracted from the leukocyte nuclei as described below (section 2.4.1).

2.3 ANALYSIS OF LATERAL CEPHALOGRAMS

2.3.1 Parameters Measured

The reason for choosing to use conventional cephalometric analysis (CCA) for this study is outlined above (Section 1.4.2.3). The rationale for the choice of variables used directly follows in that the most reliable cephalometric points in CCA determine the optimum parameters. Also for the purpose of comparison with previous studies many 'standard' parameters were chosen. Lateral cephalometry enables identification of the maxilla, mandible, cranial base and nasal bones as separate entities and various methods of their analysis, mainly in the field of orthodontics have evolved over the years.

The area of the mandibular symphysis was measured separately because of the distinction in studies on heredity between the symphysis and the rest of the mandible. Kraus *et al.* (1959) in their cephalometric study of triplets, and Garn *et al.* (1963) described the symphysis as a growth entity independent from the rest of the mandible and less susceptible to environmental modification. As such they found a higher correlation for symphyseal dimensions between parents and their children.

Similarly previous studies on inheritance of craniofacial parameters have often concluded that there are higher parent sibling correlations for intra-bony measurements than for measurements which extend beyond a single bone, (e.g. Tobias, 1955; Brash *et al.*, 1956; Krogman, 1960; and Horowitz (1963) recommended that for anthropological purposes it would be wise never to take measurements which extended beyond the limits of a single bone. Moss (1969 b) also hypothesised that since all bones including the cranial bones are biomechanically implicated in a multiplicity of functions, they consist of several skeletal units, each of which should be assessed separately. For these reasons it was felt that analysis of single bone areas should be included, and for the cranium, a subdivision of the cranial vault into its component bones, the frontal, parietal and occipital bones would be appropriate. In addition the cranial analysis lends itself well to cephalometric analysis in that the sutures on the exocranial outline are midline structures whereas the maxilla and mandible have features located laterally which reduces the cephalometric reliability. Strictly measurements taken from the endocranial surface might be more meaningful in terms of hereditary size, being relatively free from environmental influence such as muscle attachments. However it is widely recognised

that the endocranial outline and sutures cannot be reliably identified in lateral skull cephalometry (Brown, 1973).

2.3.1.1 Area Measurements

Measurement of cross-sectional area is not a standard procedure in cephalometric analysis, but has been used in a number of previous studies e.g. Nakasima and Ichinose (1984) who calculated cross sectional area on both lateral and postero-anterior cephalograms. It does not purport to measure craniofacial shape but is a very sensitive measure of size, and in conjunction with linear measurements or ratios of these measurements in the x- and y- axes shape differences between individual or mean group morphology may be implied. Area measurement can therefore be a valuable parameter in comparative studies.

The PC DIG programme allowed area measurement to be carried out and in the context of the present study six separate area measurements were chosen to augment the linear and angular cephalometric analysis devised (Figure 11). These were:

- s1 - mandibular symphyseal cross-sectional area.
- s2 - cross-sectional area of mandibular ramus and body
- s14 - cross-sectional area of maxillary outline
- s41 - cross-sectional area of cranial base outline
- s51 - cross-sectional area of nasal bones outline
- c6 - cranium outline.

In order to delineate and compute cross-sectional area on a lateral cephalogram using the PC DIG programme the points used had to be digitised sequentially. Area was automatically calculated and a figure given in square centimetres (cm^2).

2.3.1.2 Identification of Cranial sutures.

Homologous landmark identification is a recognised limitation of cephalometric analysis and there is considerable variation in the reliability of various landmarks due to the location and clarity of reproduction of their image on two dimensional radiograph film. Sutural landmarks are an obvious choice of easily defined and tangible bony landmarks but ease of identification and reliability on lateral cephalograms depends on

their position. Reliability of the frontonasal suture nasion (N) has been reported on in numerous cephalometric studies in the past. Despite its sagittal position on the bony profile in the lateral position it cannot be consistently recorded with a high degree of precision in repeatability studies (Richardson, 1966; Midtgard *et al.*, 1974; Broch *et al.*, 1981), the main reason being difficulty in its location, especially in the vertical plane.

Other sutural landmarks such as the cranial sutures between the frontal and parietal bones, (the bregmatic suture) and between the parietal and occipital bones, (the lambdoid suture), show even more variation in their ease of identification on lateral cephalograms. In the present study any attempt to characterise the outline size and shape of the skull in the frontal, parietal and occipital regions separately by using chord and subtenuce measurements was dependant on identification of the bregmatic and lambdoid sutures on the exocranial outline, points bregma (Br) and lambda (L) respectively.

Examination of the cephalograms from both the parental and control data revealed a great deal of variation in the clarity of the Br and L points on the exocranial outline from a clearly visible radiolucency to absolutely no evidence of these sutures even under optimum illumination. Cephalometric measurement of radiographs in the latter category presented a problem. This was overcome in the following manner.

A preliminary analysis of a sample of 20 lateral cephalograms on which Br and L points were clearly visible were chosen from each of the two study groups, the parental and control groups. The location of each was precisely identified by measuring to the nearest 0.25° from the S-N line and this was recorded on an acetate overlay tracing of 40 cases over a period of two consecutive days. This procedure was repeated on the same sample of 40 radiographs three weeks later and the mean value of first and second recordings taken (Table 6). Although there is a very small difference in the means of the location figure for Br and L in the parental and control groups but for validity it was felt that separate mean figures should be used for the landmark location in each of these two groups rather than use an overall mean figure which would take no account of the possibility of real differences in cranial measurements that might exist. The results of this analysis are recorded in Table 6 giving a mean figure of 82.05° for Br and 151.10° for L measured from the S-N line for the parental group, and for the control group the corresponding figures were 83.53° for Br and 151.76° for L.

Figure 11 The six area measurements used in the analysis of craniofacial morphology, all of which were used in the stepwise discriminant analysis

Table 6 Location of bregma and lambda points on the exocranial outline.

	Measurement from S-N (degrees)			
	Bregma (Br)		Lambda (L)	
	Parental	Control	Parental	Control
1	84·00	79·25	145·50	152·50
2	80·25	89·50	155·50	155·00
3	82·75	85·75	152·75	145·25
4	78·00	84·00	148·00	148·50
5	85·00	87·00	152·00	147·00
6	84·00	80·50	148·00	156·00
7	83·50	88·25	153·75	150·00
8	78·00	81·00	145·00	155·75
9	78·25	87·75	150·00	153·50
10	84·00	84·25	148·00	153·25
11	79·00	83·50	156·25	151·25
12	83·50	86·00	146·50	147·00
13	88·50	79·50	151·00	148·25
14	81·50	83·00	161·00	151·25
15	80·00	81·00	149·00	154·50
16	82·50	81·50	155·00	151·00
17	83·50	82·50	158·25	149·75
18	79·00	84·00	147·50	157·50
19	84·25	79·50	148·00	159·75
20	81·50	83·00	151·00	148·25
mean	82·05	83·53	151·10	151·76

Identification of Cranial Cephalometric Points Br and L

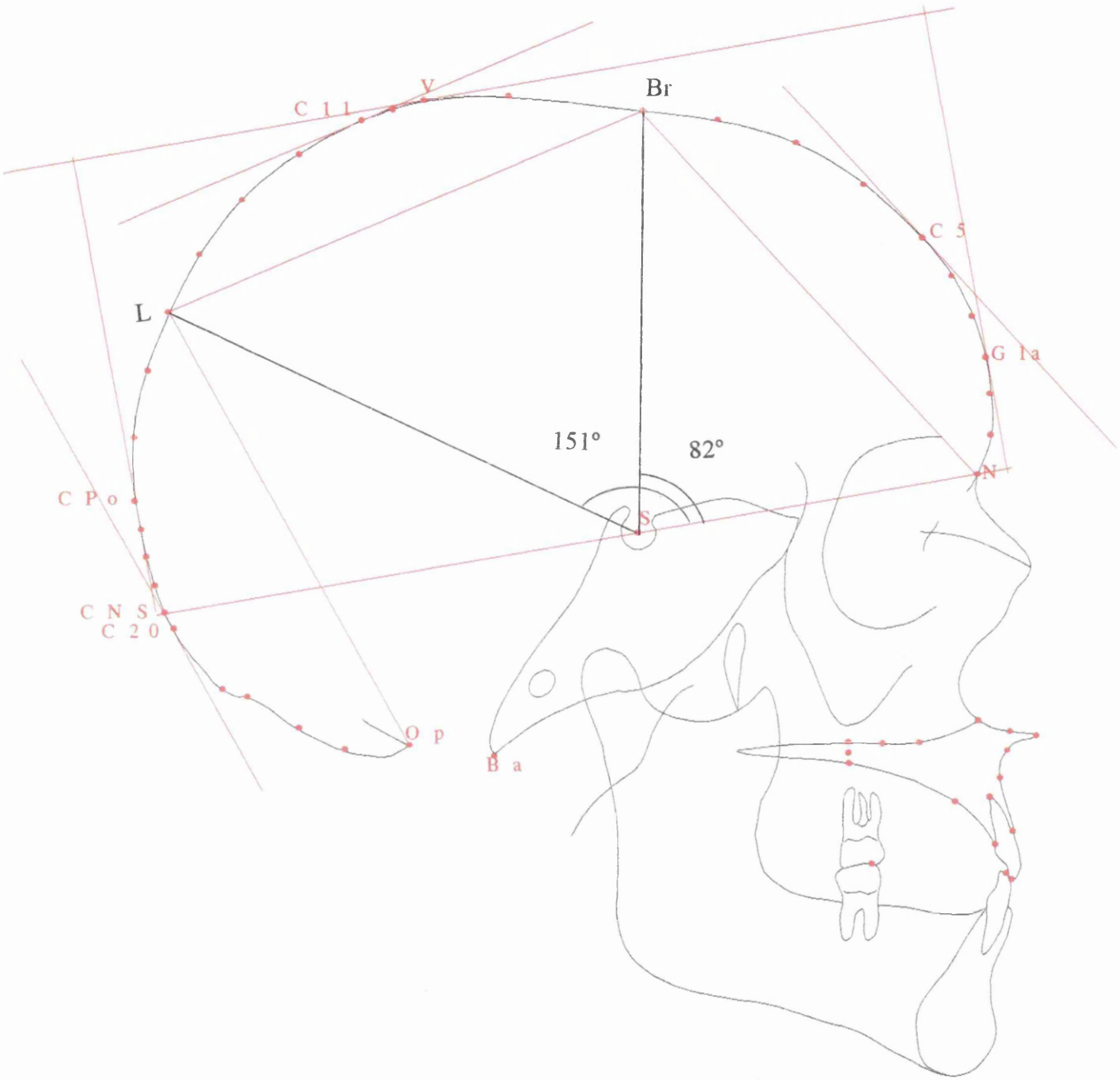


Figure 12 Identification of the bregmatic (Br) and lambdoid (L) sutures by angular measurement from the S - N line. The angular measurements used for the parental cephalometric analysis were 82° for Br and 151° for L

These figures were rounded to the nearest 0.25° and utilised in the tracing of subsequent radiographs when there was doubt about the location of these exocranial landmarks. Thus for Br and L 82° and 151° respectively were used in the parental sample (Figure 12) and 83.5° and 151.75° respectively used in the control.

2.3.2 Cephalometric Methodology

For the taking of the lateral cephalograms the patients were seated with their heads positioned in the cephalostat such that the Frankfort plane was approximately parallel to the floor. The right and left ear plugs were simultaneously positioned and the nasion rest was then moved into position. The distance from the source to mid-sagittal plane for this equipment was 152 cm. The exact position of the head was recorded by means of the vertical and horizontal scales incorporated in the nasion rest apparatus (Figure 13(a) and (b)). The film was then moved towards the right ear rod so that mid-sagittal plane to film distance was 120 mm recorded from a sliding scale attached to the film cassette holder.

Each subject was instructed to relax with his/her teeth lightly in contact and for the sake of consistency, those subjects who wore complete dentures were asked to keep their dentures in for the taking of the radiograph. The data from the head and from positioning scales were recorded as was the X-ray dosage in terms of kV, mA and exposure time before the film was exposed. Only in exceptional circumstances at the discretion of the radiographer was this varied from the standard of 74 kV, 15 mA and 0.64 s for males and 73 kV, 15 mA and 0.5 s for females (see Figure 14).

The equipment used allowed double determination of the radiographic enlargement:

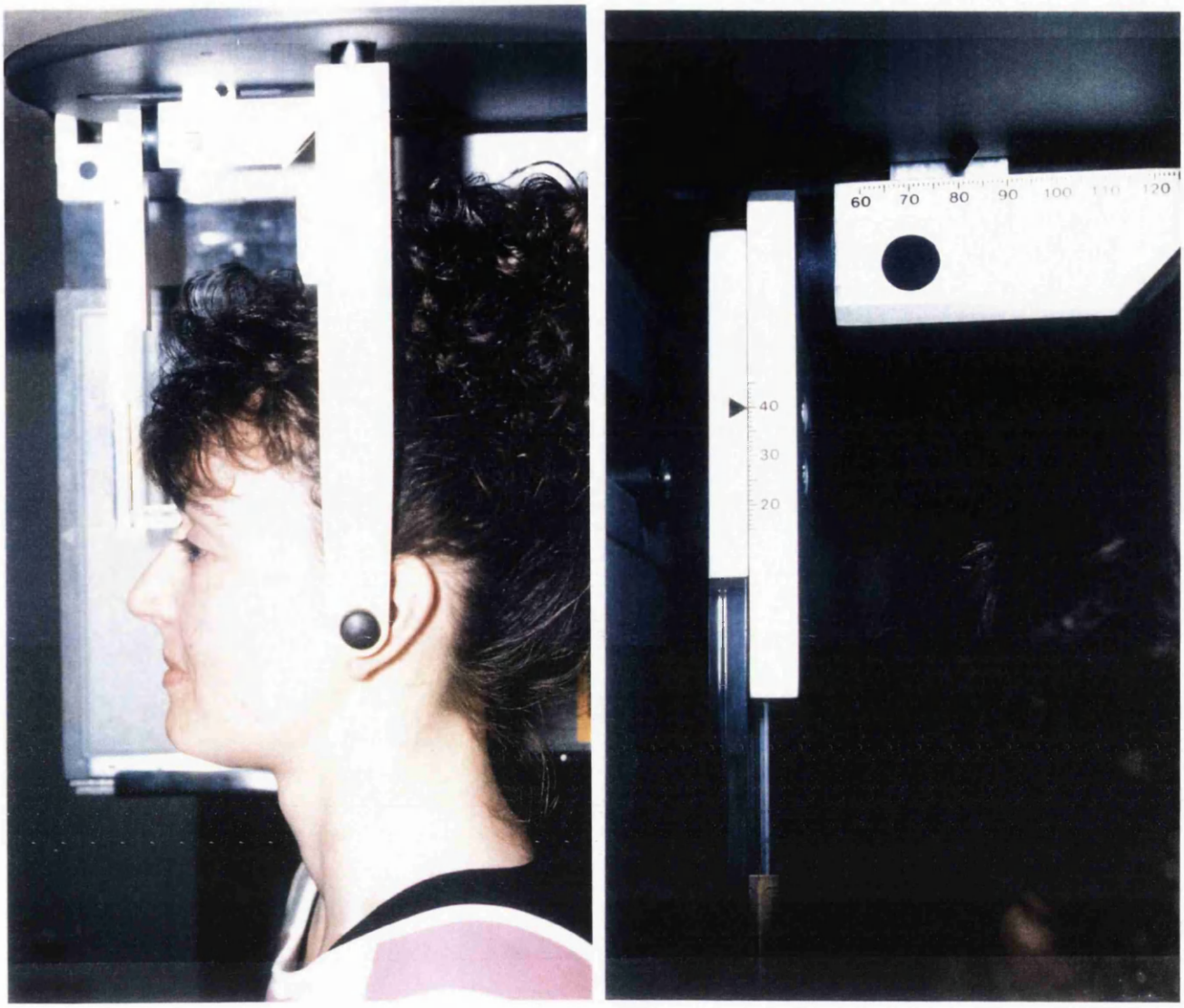
I) by means of exact measurement of the distances from X-ray source to mid-sagittal plane and mid-sagittal plane to film.

$$\text{Magnification (percentage)} = \frac{\text{Source to mid saggital plane distance}}{\text{Source to mid film distance}} \times 100$$

II) A 50 mm scale in mm gradations incorporated in the nasion rest and therefore positioned in the mid-sagittal plane, the image of which appeared in the radiographic film.

$$\text{Magnification (percentage)} = \frac{\text{Actual length mm}}{\text{radiographic length mm}} \times 100$$

The source to mid-sagittal plane distance for this equipment was 152 cm and in the vast majority of cases in this study, the mid-sagittal plane to film distance was 12 cm, giving an enlargement factor of 8%. This value approximates that which is conventionally associated with a standard cephalometer. Consequentially the measurements in this study are comparable with cephalometric norms derived from other studies e.g. Riolo *et al.* (1974) and Scheideman *et al.* (1989).



a) b)

Figure 13 a) Head positioning in the cephalostat for recording of the lateral skull cephalograms with the Frankfort plane horizontal.

b) The vertical and horizontal scale readings which recorded the position of the Nasion rest were noted for each subject.

Figure 14 The standard settings for exposure of a female subject using the Orthoceph 10, Siemens for the lateral cephalograms.

2.3.3 Methods of controlling errors

The following precautions were taken in the present study to minimise the magnitude of systematic and random error.

(a) In collaboration with the Radiography Department at Yorkhill Hospital a detailed radiographic methodology protocol was prepared in advance of the study. The relationships of X-ray target, head holder and film were predetermined and fixed. All radiographs were taken by the same two experienced radiographers. Care was taken to ensure accurate patient and ear rod positioning and each patient was advised to keep their posterior teeth in occlusion during the taking of the radiographs. A calibrated metal scale was incorporated in the cephalostat at the mid-sagittal plane. This provides permanent evidence of enlargement for each radiograph and enabled a double check of the enlargement factor. The equipment was tested using different exposures to obtain the best possible image and the film processing was of high quality. There is a conflict between radiation control and film quality in the choice of films and screens. Fast films and rare earth intensifying screens reduce the exposure greatly but give poorer definition than slower films and high definition screens. Nevertheless, exposure reduction is of primary importance and a slight reduction in image quality was sacrificed for the sake of reducing exposure time. Since the control sample was taken from existing hospital radiographs, there was no direct control over the above, but radiographs were rejected on the basis of poor quality and only those with a known magnification factor were used. Those radiographs taken using the Glasgow Dental Hospital cephalostat had the distance between the patient's mid-sagittal plane and the film recorded in the bottom right hand corner (normal range 16 - 20 mm). This enabled calculation of the magnification factor for each radiograph.

b) Landmark Identification

All the cephalograms were traced by the author in a darkened room using good quality tracing paper which did not obscure detail and a sharp hard carbon pencil. Constructed points were precisely defined and meticulously drawn on the

tracing using a range of geometric instruments. Great care was taken in the location of points that were defined as the points of greatest convexity or concavity and were identified using perpendicular or parallel lines.

c) Experimental Design

Once the landmarks had been digitised a plot was obtained utilising all the landmarks. This was done to check the resemblance of the radiographic image to the plot. A print out of all the angular, linear and area measurements was also obtained. The measurements were then checked for "wild values" against tables for normal values for similar variables. These procedures were an assessment of validity and permitted the removal of values which were not representative of the data collected and which may adversely affect the distribution of the data and the confidence intervals. The PC DIG Computer Programme used in this analysis contains a double determination facility which the author used. This function forces the user to register each image twice. During the second registration a comparison is made with the original value and if the difference is greater than a predetermined tolerance level (0·5mm in this study) an additional registration of the uncertain landmark is called for. These calls are repeated until two consecutive recordings within the 0·5mm range are achieved. Using the double determination function, each point has co-ordinates based on the mean of two acceptable registrations. Aspects of the experimental protocol designed to minimise the measurement error were as follows -

i) 25 lateral cephalograms were traced twice at the same sitting, using exactly the same fiducial points on both occasions.

ii) The computed difference between the values obtained on the first and second occasion were checked. A difference of greater than one millimetre between the two estimates was investigated. An attempt was made to redefine the points, improve the method of point location or look for an alternative more reproducible point.

iii) Steps 1 and 2 were repeated until reasonable accuracy could be expected with all points used having unambiguous definitions.

iv) All of the cephalograms were then digitised using the double determination parameter of the PC DIG Computer Programme with a tolerance of 0.5 millimetres.

v) One in five (20%) of the radiographs traced in both the parental and control samples were chosen at random using a random table method. These were traced and digitised a second time under the same conditions, but at least three weeks after the first tracing occasion.

The computer software used in the cephalometric study is known as PC DIG (McWilliam, 1989). It enables the author to devise his own cephalometric analysis using up to 100 predetermined points and will calculate any linear, angular or area measurements from the stored co-ordinates. The computer programme demanded the use of four Fiducial points for the purpose of cephalometric point orientation in the computer memory and these were recorded as a first step in the data analysis. This allowed repeated digitisation of the radiographs on separate occasions to be related to one another. The 99 selected landmarks were then traced and digitised and the sequence described using a digipad 5 digitiser (GTCO Company) which was linked to a PC Viglen 386 computer (Figure 15(a), (b) and (c)). The x- and y- co-ordinate value of each point were automatically registered in the computer memory to enable subsequent computation and statistical analysis. Plots of individual analysis and mean superimposition were produced by a Hewlett Packard ColorPro printer.

It was necessary to digitise these points in the sequence from 1 to 37 and 1 to 99 as outlined above for the cranial and facial analyses respectively to ensure that the correct parameters were being measured; and calculation of area using the PC DIG software programme requires sequential flow from the first to the last point enclosing the area to be measured.

The craniofacial morphology can be arbitrarily divided up into regions for descriptive purposes into the following;

i) Mandibular ramus body and symphysis.

ii) Facial (including maxilla).

iii) Cranial base.

iv) Nasal.

v) Cranial vault.

Maxillary and mandibular parameters are further subdivided into skeletal and dental components.

The outlines of these regions as projected onto lateral cephalograms were defined by a series of carefully chosen anatomical and constructed points (see Appendix B, part 1). Once the 99 facial and 37 cranial landmarks (Figure 16) had been digitised and stored 66 angular, linear and area parameters were computed, with parameter ratios calculated where appropriate. These measurements are detailed in Appendix B, part 2, and Table 7(a) and (b) is an abbreviated version for reference in the subsequent text. Each parameter in the facial analysis was prefixed with 's' and those in the cranial analysis prefixed with a 'c'. This facilitates reference to these variables in the subsequent text, tables and illustrations.

Finally, when producing colour plots to display results throughout the thesis a specific colour code was adopted. Red refers to parents when displayed alongside controls with no subdivision for gender or cleft type. Blue refers to males and magenta to females when gender differences are displayed, and when differentiating cleft types green refers to CL(P) and red to CP. In addition when using boxplots to illustrate results each plot displays the median, first percentile and range of measurement.

Table 7(a) Cephalometric measurements computed for facial analysis.

	Cephalometric Points	Parameter Measured (Units)
s1	Me1,Me2	Area of symphysis (cm^2)
s2	1,21	Area of mandibular ramus and body (cm^2)
s3	Rtan,Gn	Mandibular body length (mm)
s4	R1,R2	Ramus width (mm)
s5	R3,R4	Ramus height (mm)
s6	Ge,Pog	Symphyseal width (mm)
s7	Cd,Gn	Mandibular length (mm)
s8	Cd,14	Ramus length (mm)
s9	IL1,Me1	Lower dentoalveolar height (mm)
s10	3,Me1,14	Dentoalveolar height molar region (mm)
s11	Ar,Go,Me1	Gonial angle (degrees)
s12	Cd,Xi,Pm	Xi angle (degrees)
s13	IL1,AL1,Me1,14	Lower incisor angulation (degrees)
s14	Sd,32	Area of maxilla (cm^2)
s15	ANS,PNS	Palatal length (mm)
s16	ANS,26	Anterior palatal length (mm)
s17	26,PNS	Posterior palatal length (mm)
s18	Or,MPP	Maxillary height (mm)
s19	KR, MPP	Key ridge to mid palatal point (mm)
s20	ANS,Sd	Ant maxillary dentoalveolar height (mm)
s21	PNS,ANS,N	Palatal inclination (degrees)
s22	PNS,ANS,UIE,UIA	Upper incisor angulation(degrees)
s23	IIP,UMT,ANS,PNS	Occlusal plane to palatal plane (degrees)
s24	N,Me1	Anterior face height (mm)
s25	N,ANS	Upper face height (mm)
s26	Se1,PNS	Posterior upper face height (mm)
s27	S,Gn	Facial length(mm)
s28	S,Go	Posterior face height (mm)

Table 7(a) (continued)Cephalometric measurements computed for facial analysis

Variable	Cephalometric Points	Parameter Measured (Units)
s29	PNS,55	Naso pharyngeal width (soft tissue) (mm)
s30	PNS, HOR	Naso pharyngeal width (bony) (mm)
s31	N,Me1,ANS,PNS	UFH/LFH ratio
s32	S,N,A	SNA angle (degrees)
s33	S,N,B	SNB angle (degrees)
s34	A,N,B	ANB angle (degrees)
s35	ANS,PNS,Me1,14	MMPA angle (degrees)
s36	N,S,Me1,14	Maxillary plane to N-S line (degrees)
s37	N,S,Rtan,AR	S-N to ramus angulation (degrees)
s38	S,N,ANS	S-N to ANS (degrees)
s39	Or,N,S	SN to orbitale (degrees)
s40	N,S,Or,Po	SN to Frankfort plane (degrees)
s41	Se1,Se2	Cranial base area (cm^2)
s42	S,N	Anterior cranial base length (mm)
s43	N,Ba	Cranial base length (mm)
s44	S,Ba	Clivus length (mm)
s45	N,Se1	Anterior cranial base (to SE point) (mm)
s46	S,Se1	Jugum length (mm)
s47	44,47	Sella width (mm)
s48	N,S,Ba	N-S-Ba angle (degrees)
s49	Cd,56	Vertical position of condyle (mm)
s50	S,56	Horizontal position of condyle (mm)
s51	FMN,N	Area of nasal bones (cm^2)
s52	N,R	Length of nasal bones (mm)
s53	R,ANS	Maxillary/nasal bone distance (mm)
s54	R,Or	Nasal prominence to orbitale (mm)
s55	S,N,R	Nasal bone angulation (degrees)
s56	N,R,A	Nasal prominence (degrees)
s57	Or,R,N	Nasal-rhinion-orbitale (degrees)

Table 7(b) Cephalometric measurements computed for cranial analysis.

Abbreviation	Cephalometric points	Parameter Measured (Units)
c1	N-Br	Chord length of frontal bone (mm)
c2	Br-L	Chord length of parietal bone (mm)
c3	L-Op	Chord length of occipital bone (mm)
c4	V- Ba	Cranial height (mm)
c5	Gla - Po	Cranial width (mm)
c6	N1-N2	Cross-sectional area of the cranial vault
c7	Perpendicular from C5	Frontal subtenuce (mm)
c8	Perpendicular from C11	Parietal subtenuce (mm)
c9/c10	Perpendicular from C19	Occipital subtenuce (mm)

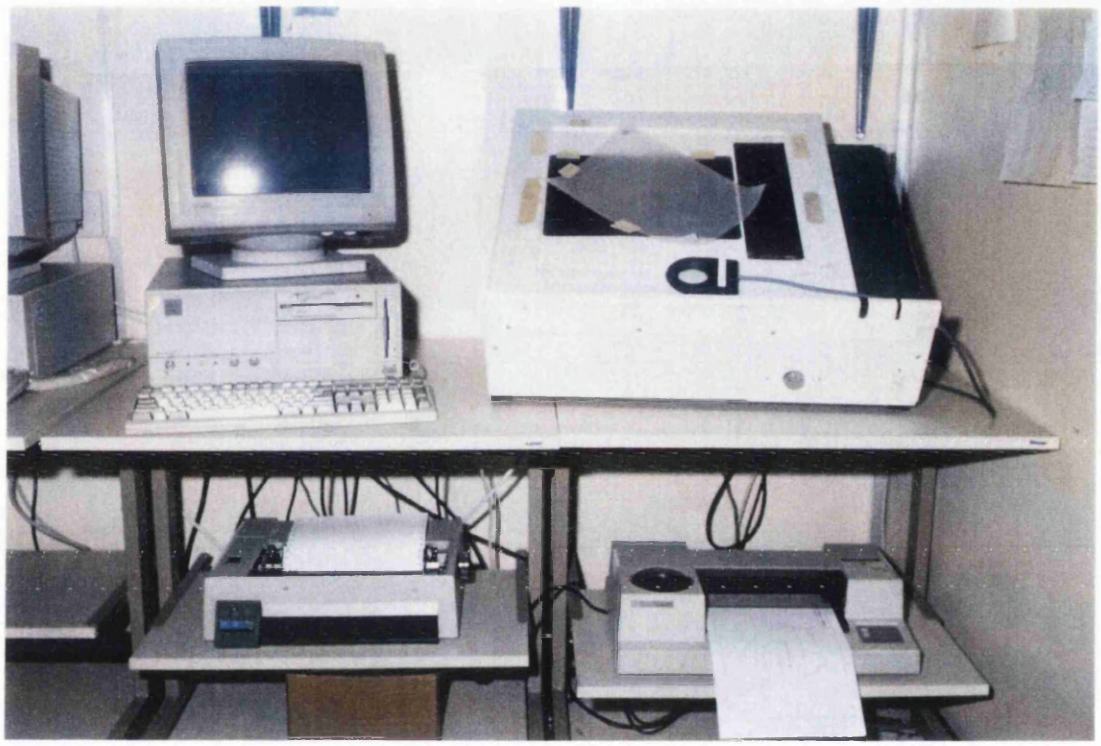
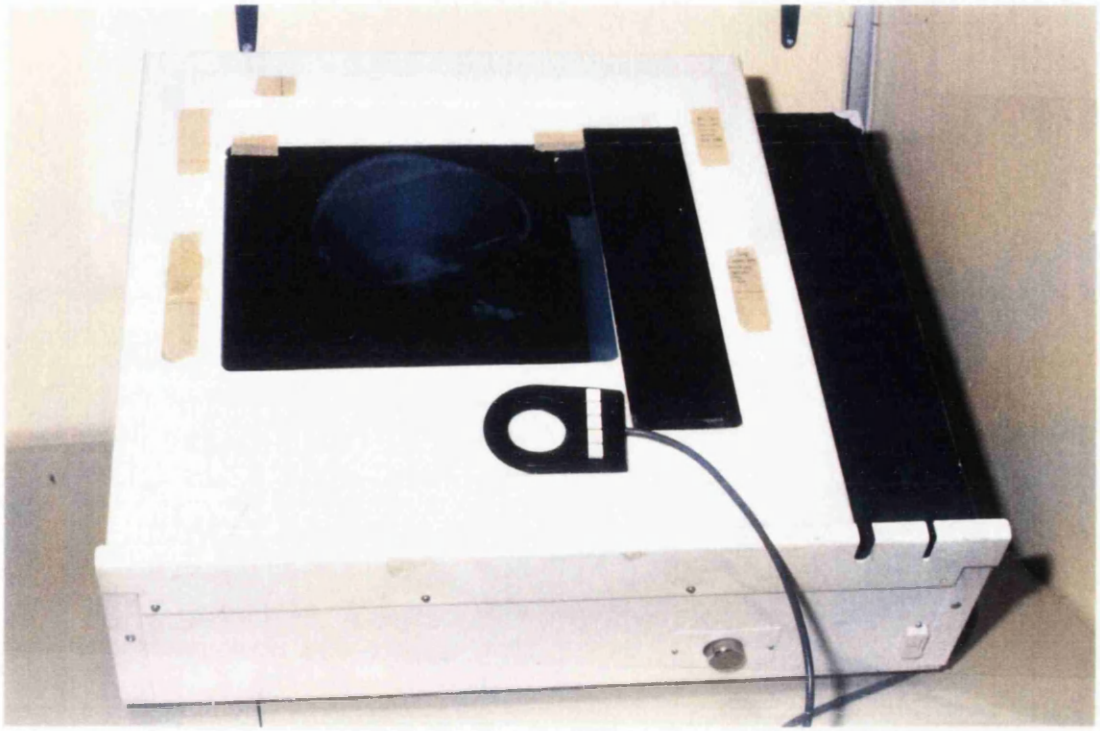
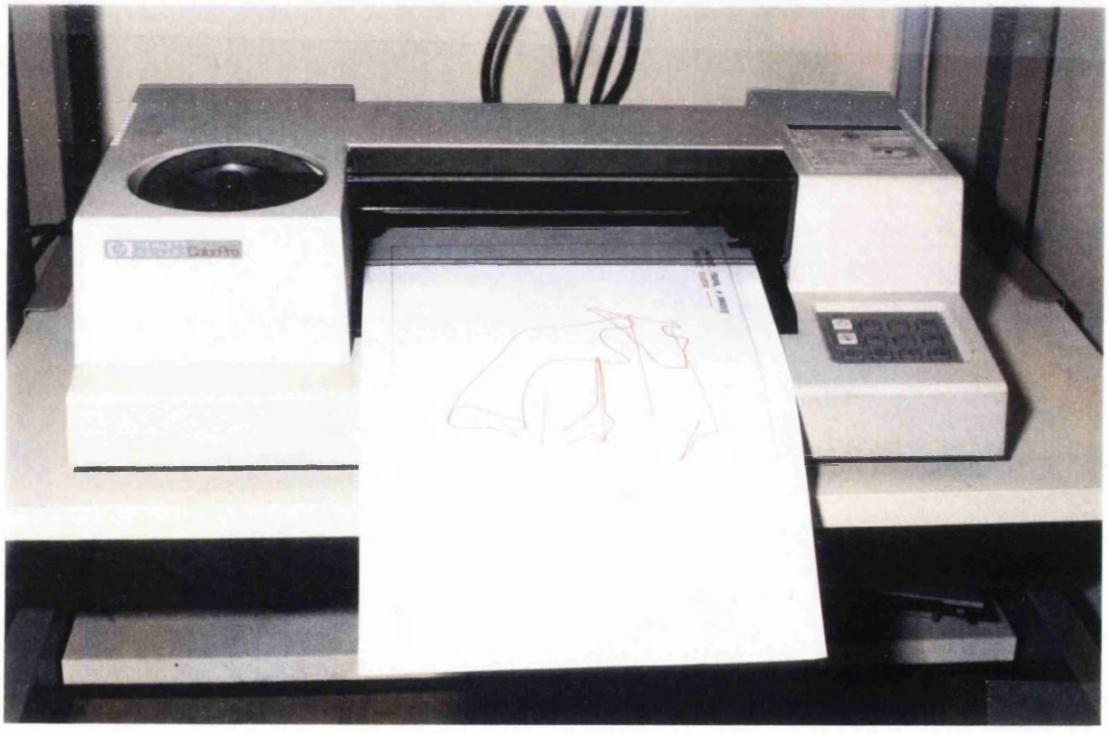


Figure 15 a) The equipment used for digitising, computation, printing and plotting of the cephalometric data.



b)



c)

Figure 15 b) Positioning of the lateral cephalogram on the GTCO Company backlit digitising screen in preparation for digitising using the digipad 5 digitiser.

c) A sample plot of a subject's facial analysis after computing and print-out on the Hewlett Packard ColorPro printer.

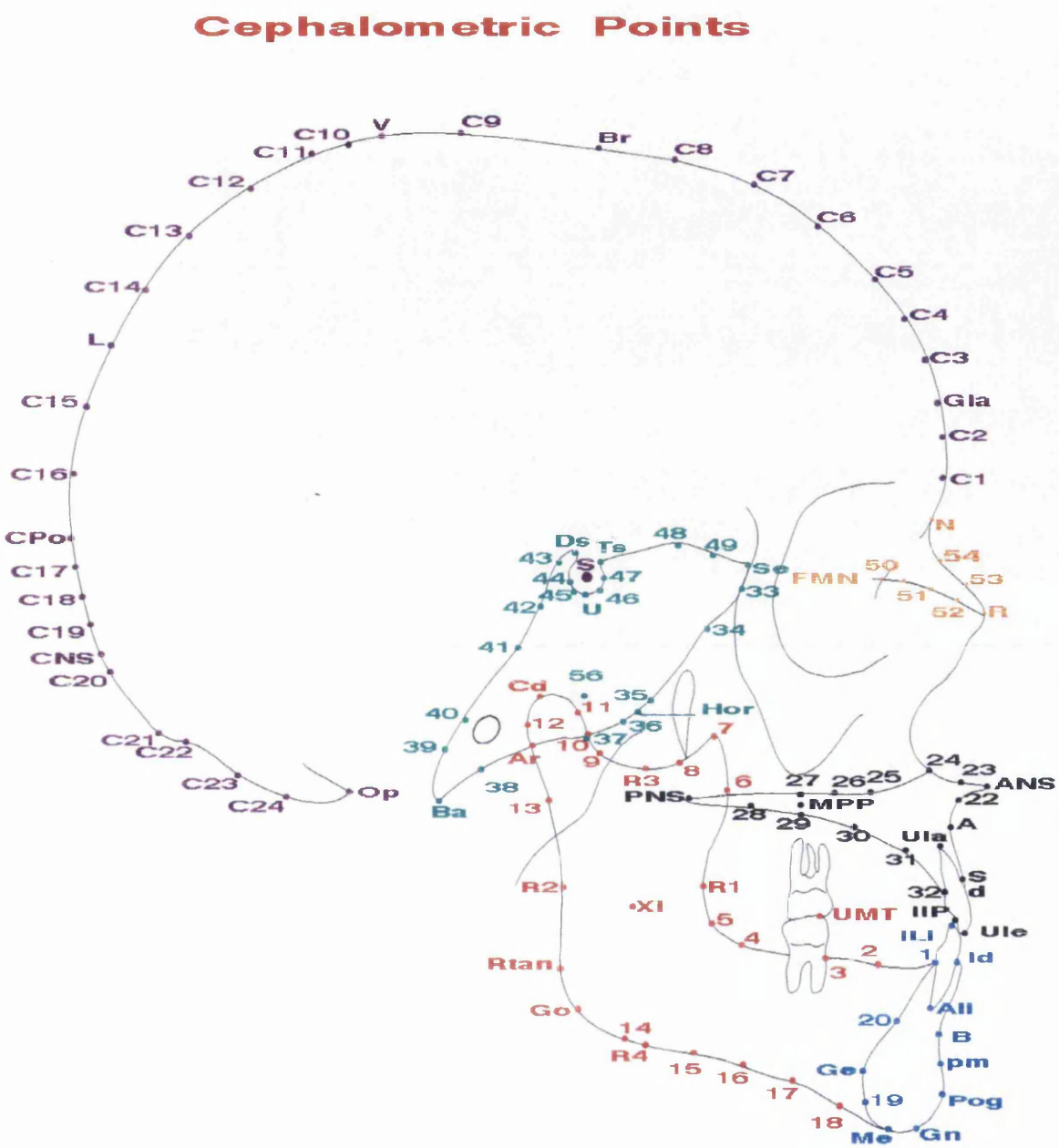


Figure 16 The 99 facial and 37 cranial points used in the lateral cephalometric analysis

The cephalometric analysis used contains both anatomic and constructed points and therefore a prerequisite to digitisation was the manual tracing of each radiograph on an acetate sheet (Figure 17). A large radius, fine line protractor was used to measure the angles used in the cranial analysis. These tracings were done under optimum conditions on a backlit screen in a darkened room. Methodologic error associated with tracing, digitising and computer plotting was assessed as follows.

I) The computer plotting error was assessed by digitising the same tracing five times. On superimposition these were found to be exact replicas and therefore there was no discernible error associated with the plotting procedure.

II) Digitisation error of the equipment was determined by digitising ten tracings selected at random (recording the fiducial followed by the 98 cephalometric points) on two consecutive occasions. Analysis of the differences indicated that the error (Dahlberg, 1940) was less than 0.02 mm in both axes. It was concluded that the error of re-establishing the co-ordinate system with this method of digitising was negligible.

III) To check tracing error or error in landmark identification, one cephalogram was traced five different times at one week intervals without reference to prior tracings. Each tracing was subsequently digitised, plotted and superimposed by the computer. These were subjectively analysed to identify any points or parameters that may be especially prone to method error.

Figure 17 Illustration of the method used to identify cranial points Gl_a, CP_0, V, CNS and C_5, C_{11}, C_{19} and C_{20}

2.3.4 Statistics protocol

The statistical analysis was carried out using the BMDP Statistical Software (1983) program p7M as follows:

I) Parental cephalometric data.

i) Systematic and random errors.

Twenty of the 94 cases in the parental sample and 20 out of 99 control cases chosen at random, were retraced and redigitised (approximately 20% of the total material in the study). The present study examined the errors associated with the measurement of cephalometric variables, as opposed to those associated with discrete points as this was considered to be more meaningful in the context of the study. The error of the linear, angular and area measurements however comprises the errors associated with all their component points.

(a) Systematic error (bias) can be checked by constructing 95% confidence intervals (95%) for the average difference between two repeated measurements made on the same individual.

(b) Random error associated with replicate measurements can be quantified by calculating the intra-class correlation co-efficient,

ii) Selection of variables for multivariate analysis

In order to carry out a multivariate analysis, the number of variables must be fewer than the number of subjects in the smallest subject group examined. Certain criteria were used to assist with the decision on which variables would be discarded and which ones would be retained for the inter-group comparisons of CP and CL(P) parents. These criteria were (a) reliability of measurement, (b) degree of correlation with another variable and (c) usefulness in light of previous studies.

iii) Adjustment of parental data.

Before embarking on a multivariate analysis, any effects due to gender, age and/or skeletal class had to be identified and taken into account. Otherwise some of the differences between the groups might have arisen simply because they were unbalanced in these respects. The samples were too small to carry out separate analyses on sub-groups e.g. males and females in each type of cleft; CP, CL and

CL(P). The alternative strategy adopted here was to adjust the data for the effects of these variables. Adjustment for gender in effect doubles the numbers for statistical analysis.

iv) Analysis of variance (ANOVA).

Differences between the means of the craniofacial variables for the CL, CLP and CP parental groups can be simultaneously analysed using a one-way ANOVA. This was carried out on the adjusted data because of the gender differences in the size of many craniofacial measurements.

iv) Stepwise discriminant analysis.

It is possible that any differences in craniofacial morphology between the various cleft groups would enable differentiation between parents of children with CP and CL(P). Stepwise discriminant analysis enables identification of those cephalometric parameters found to be most useful in the discrimination between the two groups.

II) Comparison of parental and control data

i) Standardisation of control data.

Prior to the adjustment of the cephalometric variables the same protocol for systematic and random error testing that was used for the parental data was applied. In addition to the gender effects, comparison of the parental and control groups revealed differences in age (see Figure 10) and skeletal class (see Table 4). Adjustment of these variables prior to inter-group comparison was therefore necessary and the control data was adjusted to males in Skeletal Class I .

ii) Mahalanobis distance analysis.

This is the method which was used to check if and by how much the parental data differs from the control data.

Mahalanobis distance analysis was carried out for the comparison of (a) parental and control data overall, (b) mothers and fathers versus their

respective controls and (c) intra-group comparison of skeletal class I, II and III for both parental and control groups.

iii) Two-sample t-tests

Two-sample t-tests may be used to examine the data for:

a) Differences in CL(P)/CP parents.

b) Maternal/paternal effects.

iv) Stepwise discriminant analysis.

Stepwise discriminant analysis identifies those parameters found to be most useful in the differentiation between two groups. In the context of this study it is important that paternal and maternal differences are analysed separately.

III) Genetic Data Analysis

Table 5 shows the distribution of cleft types for the 76 unaffected parents and 19 probands who volunteered to participate in the study. In the absence of control data for the West of Scotland population, the control data for the present study was derived from a different but ethnically similar population. Use of such data can be justified by the fact that allele frequency in control subjects in populations as diverse as Australia, America and Britain were found to be remarkably similar. Hence the allele and genotype frequency figures quoted in the UK study by Holder *et al.* (1992) are used for the present comparison (Table 8). The raw genotype data is presented in Appendix A.

i) Chi-squared tests.

The differences between parental, proband and control allele frequencies (Table 9) and genotype frequencies (Table 10) were evaluated by Chi-squared analysis. Likewise the genotypes for parents and probands in both cleft groups were recorded and tabulated alongside the Holder *et al.* (1992) control data and the chi-squared test applied.

Table 8 TGFα allele frequencies from previous population controls.

Author	Allele frequency					
	A1	A2	B1	B2	C1	C2
1. Murray *et al.*, (1986)	0·190	0·810	0·290	0·710	0·940	0·060
2. Ardinger *et al.*, (1989)	0·130	0·870	0·270	0·730	0·950	0·050
3. Chenevix-Trench *et al.*, (1991)	-	-	-	-	0·945	0·055
4. Holder *et al.*,* (1992)	0·130	0·870	0·360	0·640	0·960	0·040
5. Qian *et al.*, (1993)	0·076	0·924	0·293	0·707	0·930	0·070
6. Sassani *et al.*, (1993)	-	-	0·262	0·738	0·911	0·089

Control Characteristics:

1. American Caucasian n = ?
2. American Caucasian, n = 102
3. Australian Caucasian, n = 100
*4. British Caucasian, n = 62
5. Alcasian Caucasian, n = 99
6. American Caucasian, n = 98

*Control data used in the present study.

Table 9 Number of chromosomes with TGFα alleles, and allele frequencies in probands and controls classified according to clefting status- CP and CL(P), and in controls.

Group		TGFα allele count and (frequency)					
		A1	A2	B1	B2	C1	C2
CP	Probands	2 (0·125)	14 (0·875)	4 (0·222)	14 (0·778)	13 (0·812)	3 (0·188)
	Parents	15 (0·250)	45 (0·750)	18 (0·310)	40 (0·690)	53 (0·855)	9 (0·145)
CL(P)	Probands	1 (0·050)	19 (0·950)	7 (0·318)	15 (0·682)	15 (0·834)	3 (0·166)
	Parents	6 (0·080)	68 (0·920)	25 (0·338)	49 (0·662)	68 (0·872)	10 (0·128)
	Control*	16 (0.130)	108 (0.870)	34 (0.360)	77 (0.640)	115 (0.960)	5 (0.040)

* Holder *et al.*, (1992)

Table 10 Number of individuals presenting with each genotype produced by restriction enzyme digestion with *Bam*HI, *Rsa*I and *Taq*I.

Genotype	CP				CL(P)				Control
	♂	♀	Parents	Probands	♂	♀	Parents	Probands	No.
*Bam*HI									
A1 A1	0	0	0	0	0	0	0	0	0
A1 A2	5	10	15	2	0	6	6	1	17
A2 A2	7	8	15	6	16	15	31	9	45
*Rsa*I									
B1 B1	1	1	2	0	1	3	4	0	10
B1 B2	4	9	13	4	8	7	15	7	22
B2 B2	7	7	14	5	9	10	19	4	28
*Taq*I									
C1 C1	9	13	22	5	13	16	29	6	55
C1 C2	4	5	9	3	3	5	8	3	5
C2 C2	0	0	0	0	2	0	2	0	0

♂ = Fathers ♀ = Mothers

Finally the genotype frequency data were further subdivided into maternal and paternal for each cleft subgroup, so that any gender effect within the parents could be examined using the chi-squared analysis.

ii) Relative risk analysis

In those instances where the χ^2 test revealed a significant difference in allele or genotype frequency between parental and control groups the relative risk (R) and it's 95% C.I. was calculated according to the method of Woolf (1955).

iii) Logistic regression analysis

A logistic regression model can be used to determine which genotypic markers, if any, best distinguish between the parental and control groups

A second logistic regression model using parental data only and taking cleft group plus four additional variables namely gender and the genotypes derived from each of the three restriction enzymes may be used to determine which genotype or genotype/gender combination interactions best distinguishes between CP and CL(P) groups.

IV) Analysis of combined phenotypic/genotypic data .

i) Analysis of variance

This enables analysis of phenotypic variance with the various genotypes in the parents of CP and CL(P) children simultaneously. Separate ANOVA analysis for mothers and fathers is necessary because of gender differences in the size of many craniofacial parameters. It was feared that combining the data might well mask a significant gender effect in this genotype/phenotype analysis. The cephalometric data was adjusted for age and skeletal class as was done prior to the discriminant analysis earlier in the study.

ii) Logistic regression analysis

Logistic regression can also be used with the inclusion of craniofacial variables as well as genotype, the craniofacial variables being chosen for their ability to discriminate between CP and CL(P) parents. This genotype/phenotype model should maximise discriminative power in the search for markers which can be used to predict parental predisposition to producing a child with either a CP or CL(P) deformity.

2.3.5 Choice of cephalometric linear and angular variables

The decision to use a discriminant analysis meant that the 67 parameters originally chosen needed to be reduced. The choice of a reduced number of cephalometric points and variables was determined by four main factors;

i) The need to describe as comprehensively as possible all anatomic regions of the head and face represented on the lateral head plate, with particular attention to areas thought to be of interest in the context of heredity of cleft lip and palate.

ii) The desire to use parameters which were defined by landmarks that were easily identified and could be reliably reproduced.

iii) The desire to use, as far as possible, larger rather than smaller measurements to reduce the significance of minor errors in landmark identification.

iv) The need to avoid unnecessary duplication or the use of highly correlated variables in the analysis (defined as pairs of variables with r values greater than 0.75). The latter factor is a prerequisite for the efficient application of multivariate analysis.

When two variables were highly correlated ($r > 0.75$), the choice of which one to eliminate was based on;

a) the perception of the actual anatomic information contained in the variable

b) the reliability with which it could be measured and

c) the desire to use variables that other investigators had used in similar studies.

For example, when measuring mandibular ramus length articulare to gonion (Ar-Go) and condylion to gonion (Cd-Go) are highly correlated. Articulare is a reasonably reproducible landmark whereas condylion is often obscured by the shadows of the clivus and petrous part of the temporal bone.

It is accepted that condylion to gonion does give a truer estimate of the anatomical length of the ramus, as articulare, being defined as the intercept of two shadows, has no anatomical significance. Also a study of the reproducibility of condylion (Forsberg, 1989) concluded that it was an acceptably accurate point in cephalometric analysis. Cd-14 (s8) was therefore retained in preference to Ar-Go as a measure of mandibular ramus length.

Dentoalveolar measurements from edentulous individuals produced extreme values which obscured the pattern of data for these measurements. It was decided to remove these cases, leaving just 83 subjects, 42 mothers and 41 fathers. The difficulty in identification and subsequent unreliability of nasal measurements in a large number of cases led to the exclusion of these variables. In addition, one of each pair of highly correlated variables (correlation > 0.75) was removed, except in cases where both measurements were considered to be crucial (see Table 11). For example angles S-N-A and S-N-ANS were highly correlated (r = 0.87). The S-N-ANS was discarded because point A is more reliable in the horizontal plane than point ANS and therefore angle S-N-A is the more accurate measurement of maxillary prominence. The linear measurements S-Go (posterior face height) and Cd-14 (ramus height) were also highly correlated (0.84). Both of these were retained, however, because it was felt that each made a different but essential contribution to the analysis.

Also total anterior face height (N-Me, s24) and facial length although highly correlated (S-Gn, s27) were both retained because previously published studies (Coccaro *et al.*, 1972; Nakasima and Ichinose, 1983; Prochazkova and Tolarova 1986) have indicated that both are important in differentiating relatives of CL(P) subjects from normal control populations. Nine parameters were chosen to describe the cranial morphology (Figure 18) and 28 parameters were chosen as the optimum area (see Figure 11), linear (Figure 19) and angular (Figure 20) measurements for analysis of the facial structures (Table 12).

Table 11 Most highly correlated pairs of variables (r > 0·75)

Variable pair		Correlation	Parameters Removed
s56	s54	0·936	Both
s45	s46	0·930	Both
s27	s7	0·918	Neither
s38	s32	0·871	s38
s36	s35	0·856	s36
s52	s56	0·855	Both
s8	s28	0·854	s28
s42	s45	0·852	s45
c5	c6	0·841	Neither
s29	s30	0·829	s29
s24	s27	0·827	Neither
s11	s12	0·813	s12
s52	s51	0·810	s52
s8	s2	0·803	Neither
s28	s2	0·801	s28
s24	s9	0·795	s9
s33	s32	0·794	Neither
c4	c8	0·785	Neither
s43	s42	0·770	s43
c9	c10	0·759	One or other included
s56	s55	0·766	Both

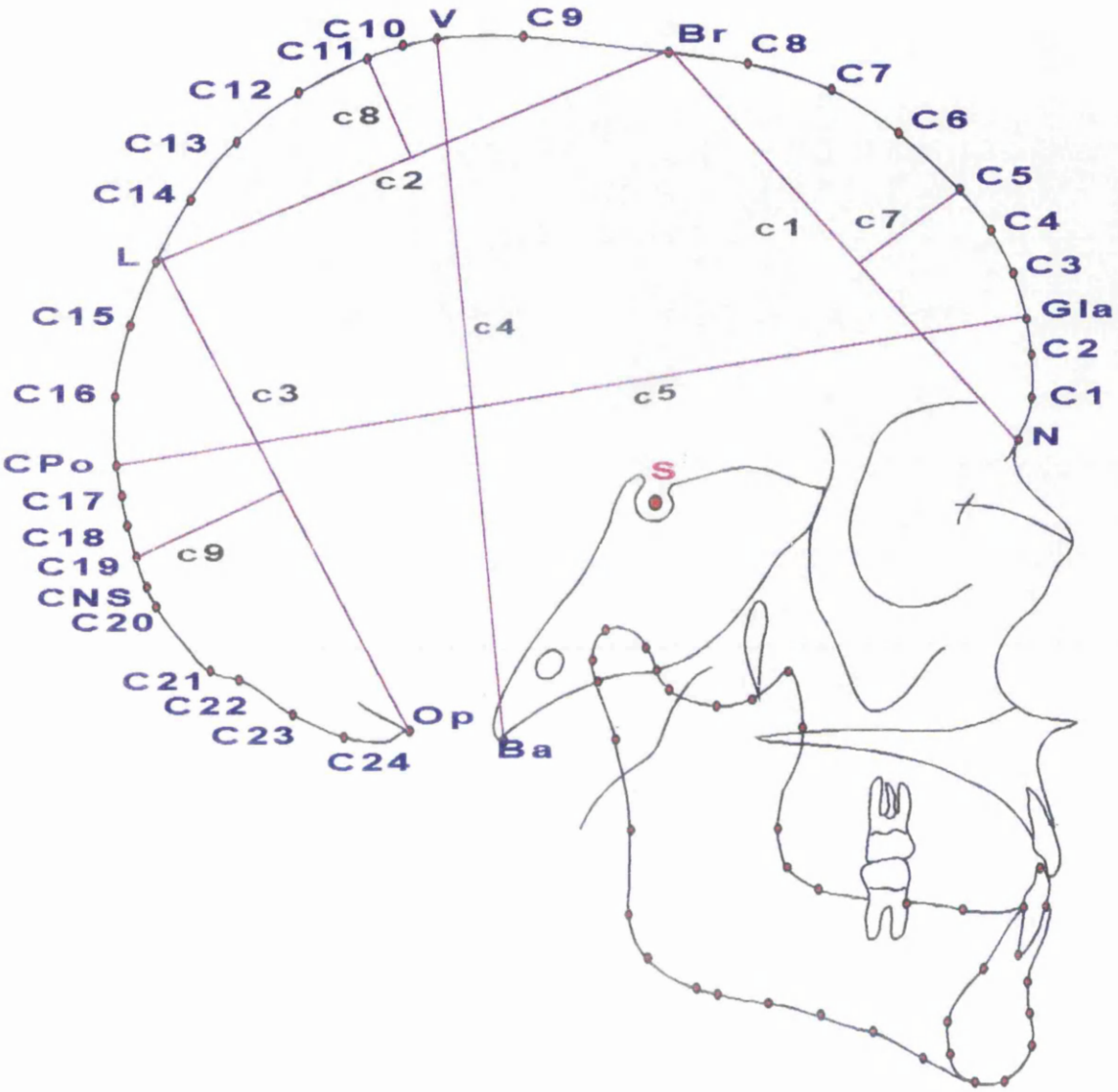


Figure 18 The eight linear measurements describing the cranium which were used in the stepwise discriminant analysis

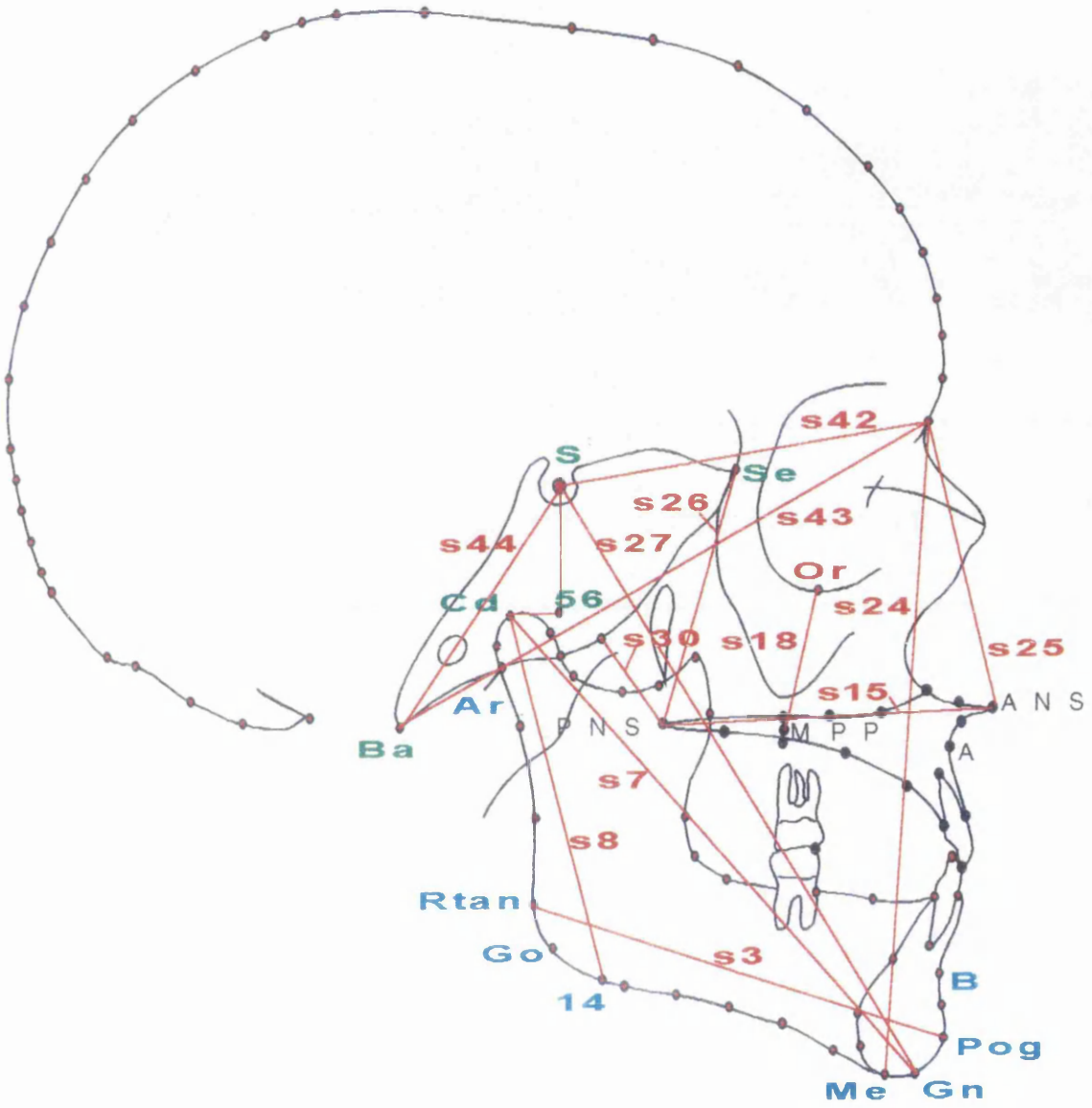


Figure19 The 14 linear measurements describing the facial bones and cranial base which were used in the stepwise discriminant analysis (Sella width (s47) not included in the diagram)

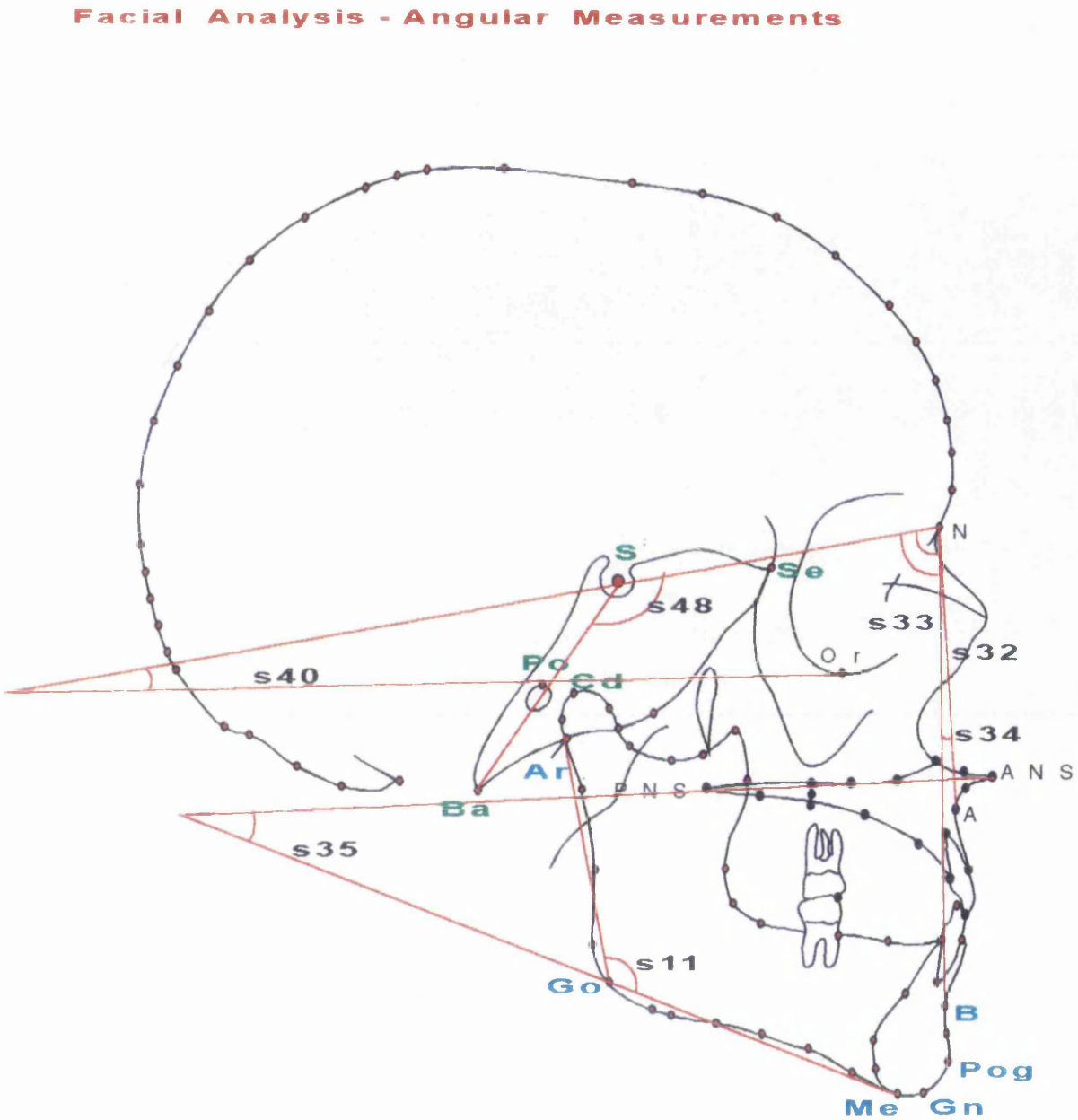


Figure 20 The seven angular measurements chosen for the analysis of the facial bones and cranial base in the stepwise discriminant analysis (S - N - Or (s39) not included in the diagram)

Table 12 Thirty seven cephalometric measurements used in discriminant analysis

(a) Six area measurements	
s1	Area of Symphisis
s2	Area of Mandible
s14	Area of Maxilla
s41	Area of Cranial Base
s51	Area of Nasal Bones
c6	Area of Cranium

(c) Seven angular measurements	
s11	Gonial Angle
s32	Maxillary Prominence
s33	Mandibular Prominence
s35	MMPA
s39	Orbital Prominence
s40	S-N to FP Angle
s48	Cranial Base Angle

(b) 23 Linear measurements	
(i) Facial and cranial base	
s3	Body Length
s7	Mandibular Length
s8	Ramus Length
s15	Palatal Length
s18	Maxillary Height
s24	Anterior Face Height
s25	Upper Face Height
s26	Posterior Face Height
s27	Facial Length
s30	Naso-pharyngeal Depth
s31*	Face Height Ratio
s42	Anterior Cranial Base Length
s44	Total Cranial Base Length
s47	Sella Width
s49	Condylar Position - Horizontal
s50	Condylar Position - Vertical
(ii) Cranial	
c1	Frontal Chord
c2	Parietal Chord
c4	Occipital Chord
c4	Cranial Height
c5	Cranial Width
c7	Frontal Subtenuce
c8	Parietal Subtenuce
c9/c10	Occipital Subtenuce

* UFH:LFH ratio measurement.

2.4 DNA ANALYSIS

2.4.1 DNA extraction from blood samples

Fresh blood samples in EDTA as anticoagulant from the parents and some of the probands (see Table 5) were received in the laboratory within twenty four hours of having been taken. DNA was extracted and quantified using standard methods (Sambrook *et al.*, 1989).

2.4.2 Primer preparation and deprotection

Qian *et al.* (1993) provided the technical data for identification of the TGFα gene which includes 66 nt of intron V and 390 nt of exon VI. The fact that this fragment detects the *Bam*HI, *Taq*I and *Rsa*I RFLPs indicates that these three polymorphisms are located close to one another in a region which encompasses the intron V-exon VI junction. Qian *et al.*, (1993) characterised these three sites by PCR and provided the technical data which enabled construction of the PCR primers.

The preparation of the three primer pairs used in this study was carried out in the laboratory using a 391 DNA Oligosynthesiser (Applied Biosystems Incorporated) as per the ABI user guide.

2.4.3 Amplification of genomic DNA

This was carried out using a process known as the polymerase chain reaction (PCR) (Figure 21). PCR is an in vitro method of oligonucleotide synthesis by which a specific segment of DNA in this case the TGFα region on chromosome 2p13 was amplified. The standard PCR method as described by Kogan *et al.* (1987) was used.

The PCR reaction required appropriate concentrations and volumes of the primers, deoxynucleotide phosphates (dNTP), a standard buffer, the parental DNA, *Taq* DNA polymerase and water to make up a 50 µl aliquot.

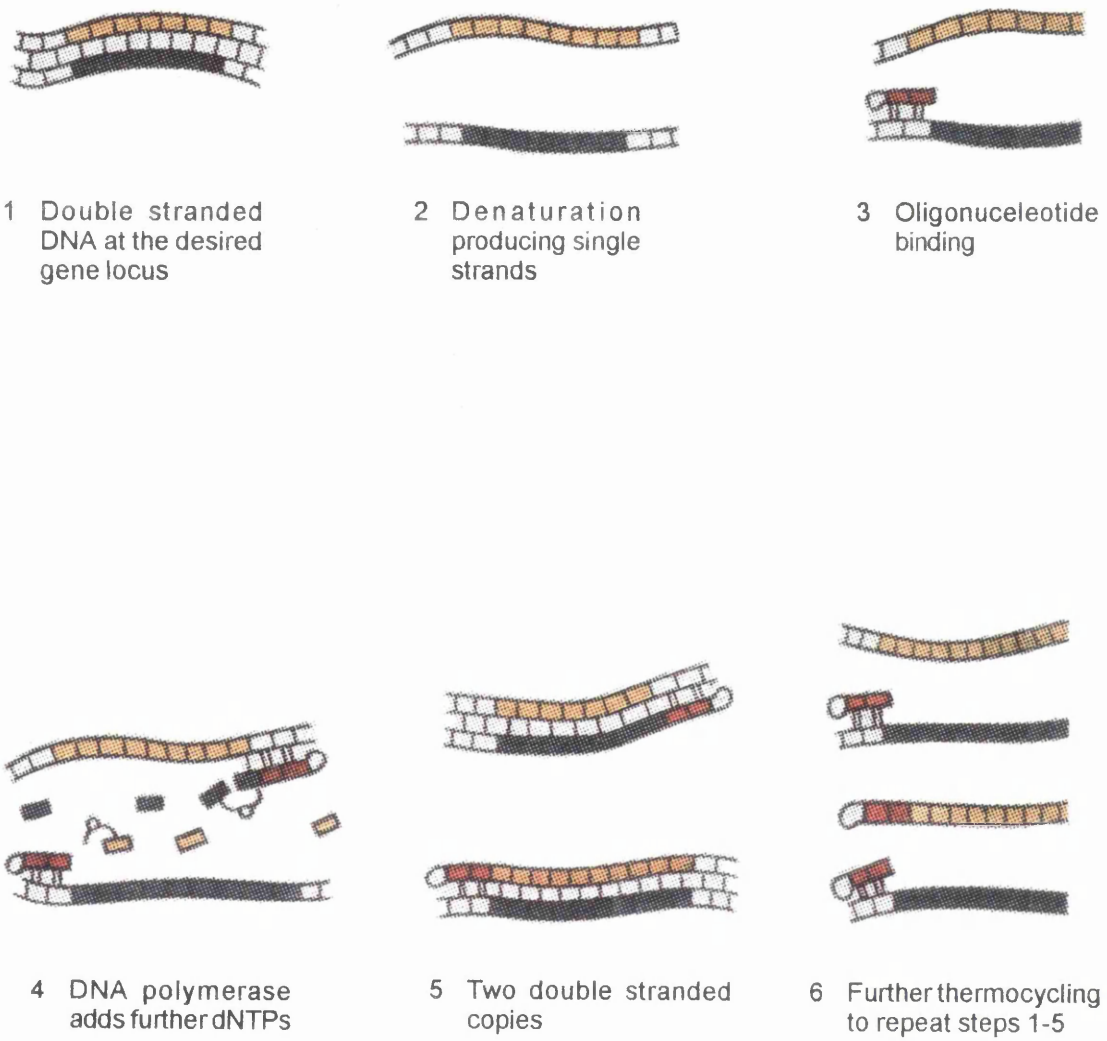


Figure 21 The polymerase chain reaction (PCR) used for the amplification of genomic DNA. A sequence of denaturation, primer hybridization and primer extension under the influence of DNA polymerase

The following procedure was followed in the preparation of a 50 µl PCR reaction mixture for each DNA sample.

1. Appropriate amounts of each of the primers, buffer, dNTPs and water were added, mixed thoroughly using a vortex and subjected to five minutes exposure to ultraviolet light to eliminate any impurities.
2. An appropriate volume of DNA which varied according to the concentration was added.
3. Finally 0.5 µl of *Taq* was added, and the mixture vortexed and spun briefly to remove liquid from the tube walls.
4. 75 µl of mineral oil was layered over each reaction mix to prevent evaporation during the thermal cycling.

Figure 22 illustrates the Perkin Elmer Cetus thermal cycling instrument which was programmed to provide the optimum conditions for each of the primer pairs in turn. Repeated cycles of heat denaturation of the DNA, annealing of the primers to their complimentary sequences, and extension of the annealed primers using DNA/*Taq* polymerase (Boehrringer).

2.4.4 PCR product checking and restriction enzyme digestion.

Before proceeding to restriction enzyme digestion of the amplified TGF-a fragment of the DNA, a five µl sample of the PCR product was transferred into a fresh eppendorf tube, mixed with five µl of loading mix, vortexed and transferred to an agarose gel for electrophoresis, (Figure 23).

Digestion of PCR product with *Bam*HI *Rsa*I and *Taq*I restriction enzymes was then carried out.

Table 13 indicates the sequence and location of the polymorphic restriction sites for the three enzymes used on the TGFα gene and Figures 24, 25 and 26 are samples of the banding patterns after gel electrophoresis.

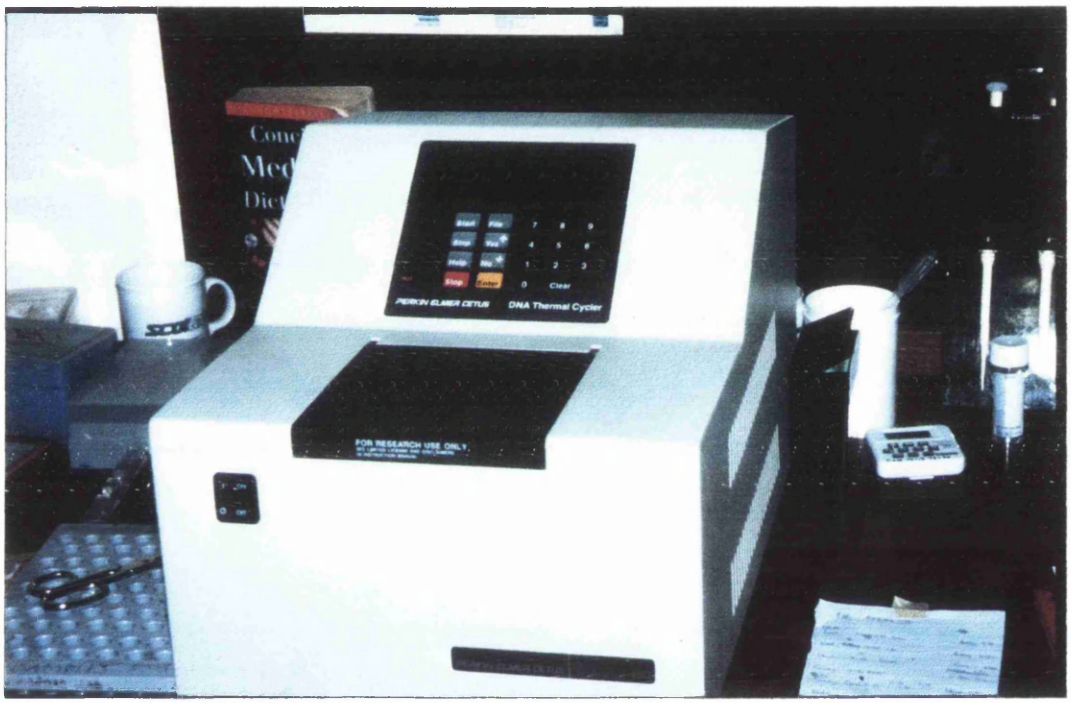


Figure 22 The Perkin Elmer Cetus PCR machine used to amplify the TGFα locus in the parental DNA

PCR Product Check Gel

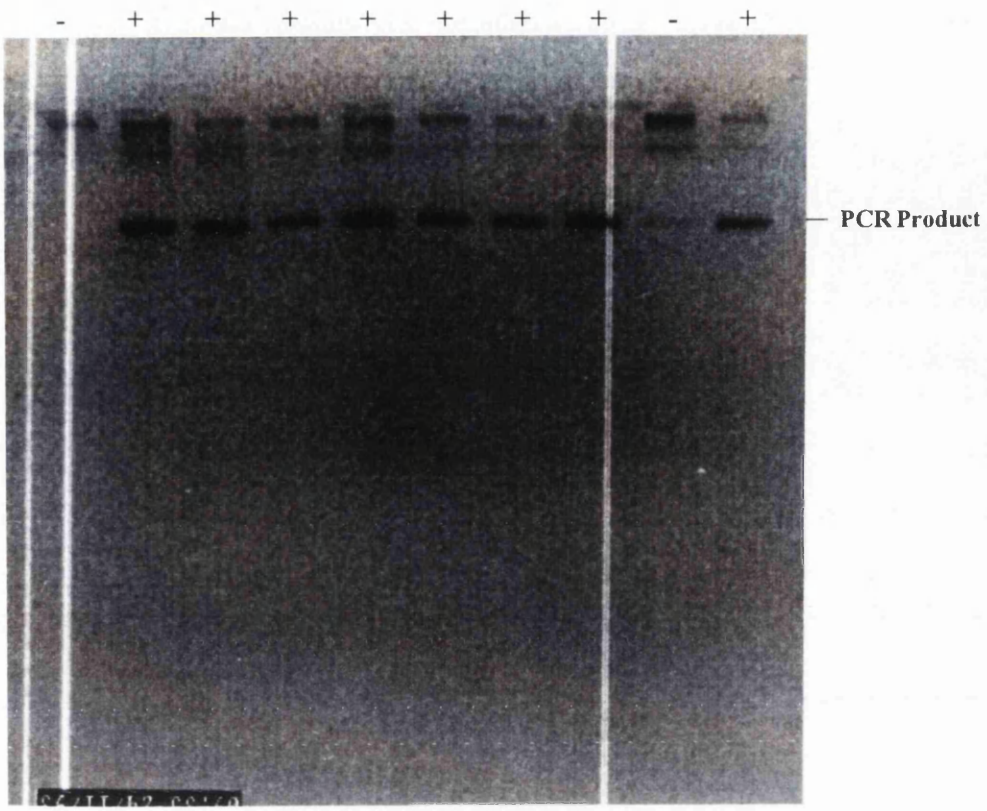


Figure 23 - Electrophoresis checking gel

Checking for PCR product prior to restriction enzyme digestion in a sample of nine subjects in the study.

+ = PCR product present
- = PCR product absent

Table 13 Polymorphic sequences and primer pairs used for the PCR amplification

Polymorphic allele	Primer pair	Size of PCR fragment (bp)	Polymorphic Restriction Sites (s)	
			Sequence	Location
A1 (10 kb) A2 (7 kb)	P1 P2	434	AGCATTGGCTCCCTCTGC - - - - - - - - A - - - - - - - - - *Bam*HI	Exon V1 (in the 3'UTR, at 2,767 bp from the first ATG)
B1 (1·5 kb) B2 (1·2 kb)	P3 P4	657	ACTGAAAGTATTATGTCA - - - - - - - - - - C - - G - - - - *Rsa*I	Intron V (177 bp upstream of the acceptor site of exon VI)
C1 (3 kb) C2 (2·7 kb)	P5 P6	662	AGGTCTCTAATGACCTTA - - - - - - - - - - - - - - - - - - *Taq*I	Intron V (1,602 bp upstream of the acceptor site of exon VI)

2.4.5 DNA band separation and recording.

Standard methods were used to separate, identify and purify DNA fragments using horizontal electrophoresis. Nusieve agarose gels (2·5%) stained with ethidium bromide were used, and standard methods of gel preparation, electrophoresis and transillumination photography according to the procedure outlined by Sambrook *at al.* (1989)

Bam HI

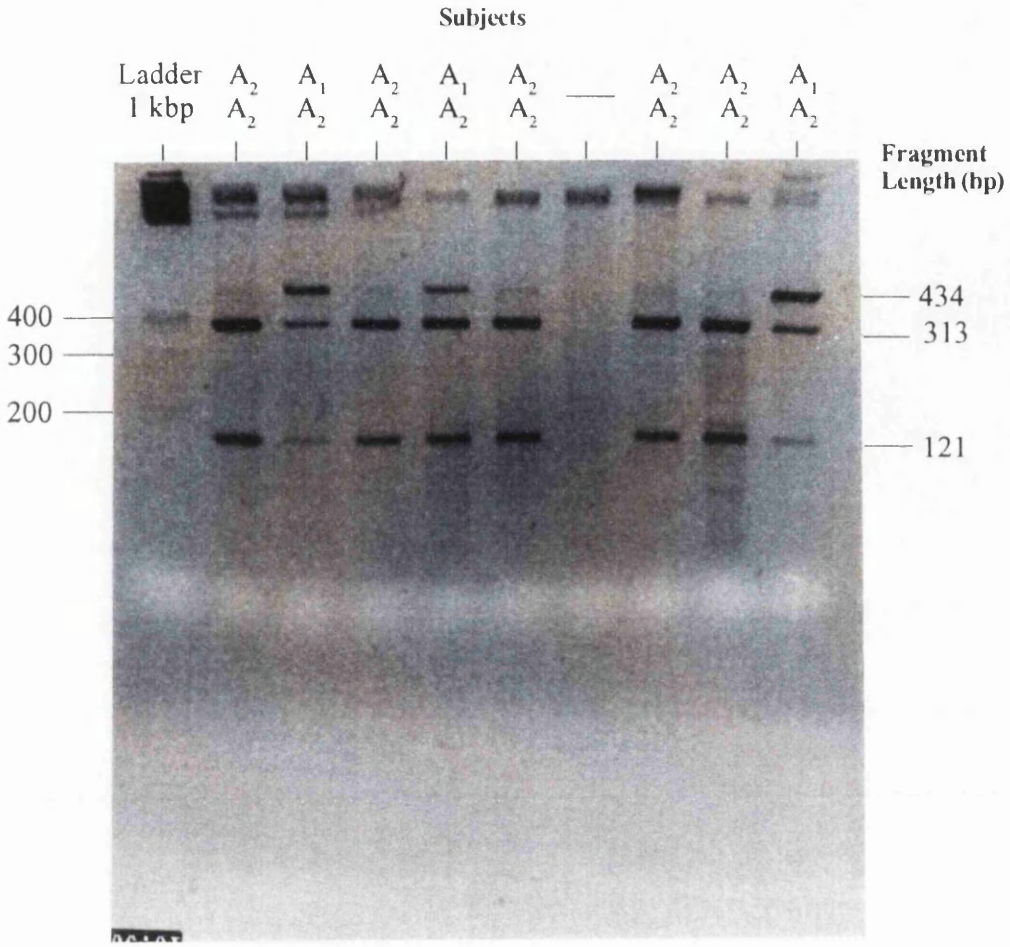


Figure 24 - Electrophoresis gel from a sample of nine subjects after digestion with Bam HI

The PCR amplified product with the A_2 allele present is cleaved by Bam HI, giving two fragments of 121bp and 313bp (eg. lane 1), whereas digestion of the PCR product in the presence of the A_1 allele results in no cleavage giving a band at 434bp (eg. lane 2). No example of homozygosity for the A_1 allele is shown, as the $A_1 A_1$ genotype was unrepresented in the entire sample of parents and probands in the present study.

Rsa I

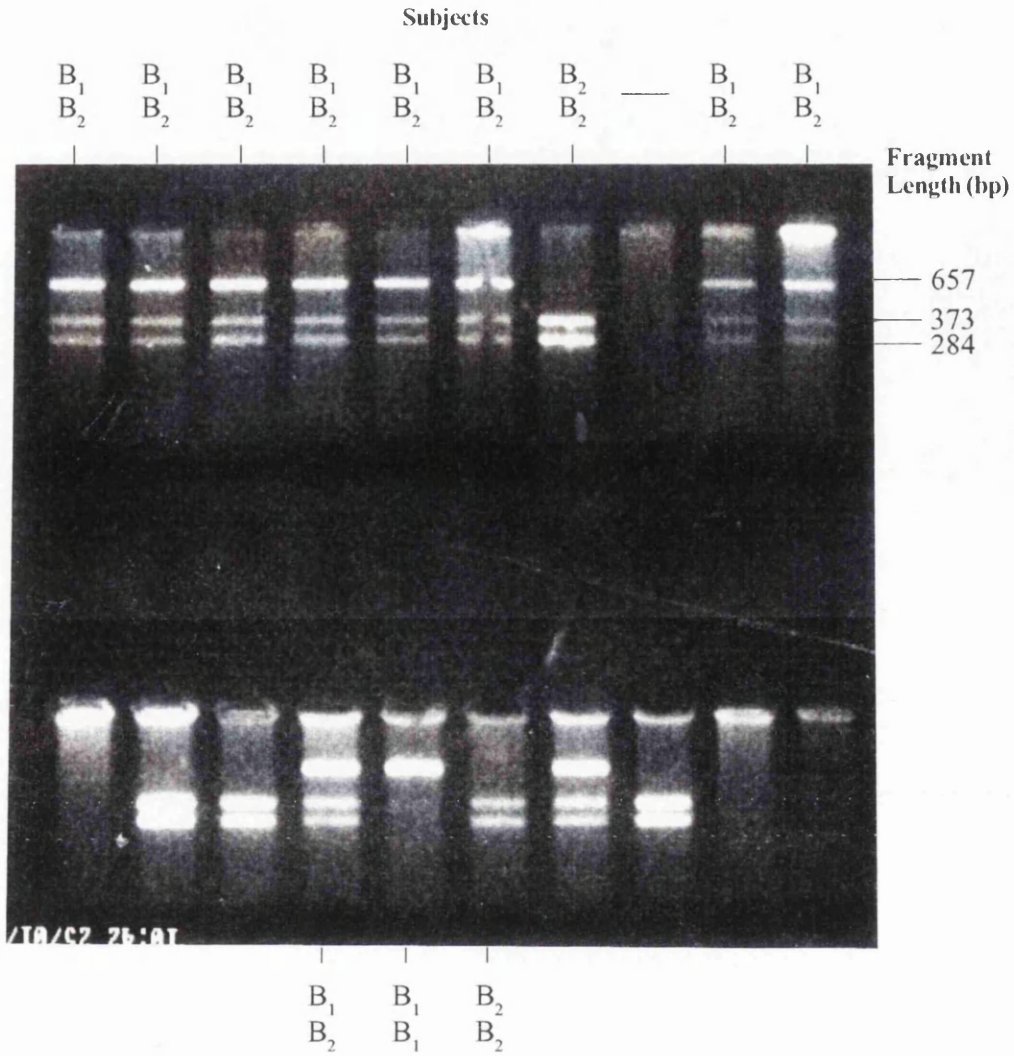


Figure 25 - Electrophoresis gel after digestion of PCR product from a sample of 20 patients with Rsa I

In the absence of the Rsa I restriction site, the 657 bp fragment is not cleaved by this enzyme. However, when the B_2 allele is present, cleavage of the 657 bp fragment into two fragments of 373 and 284 bp does occur. This explains the banding pattern shown above; and no other polymorphism has been detected in the 657 bp two-allele specific PCR products.

Taq I

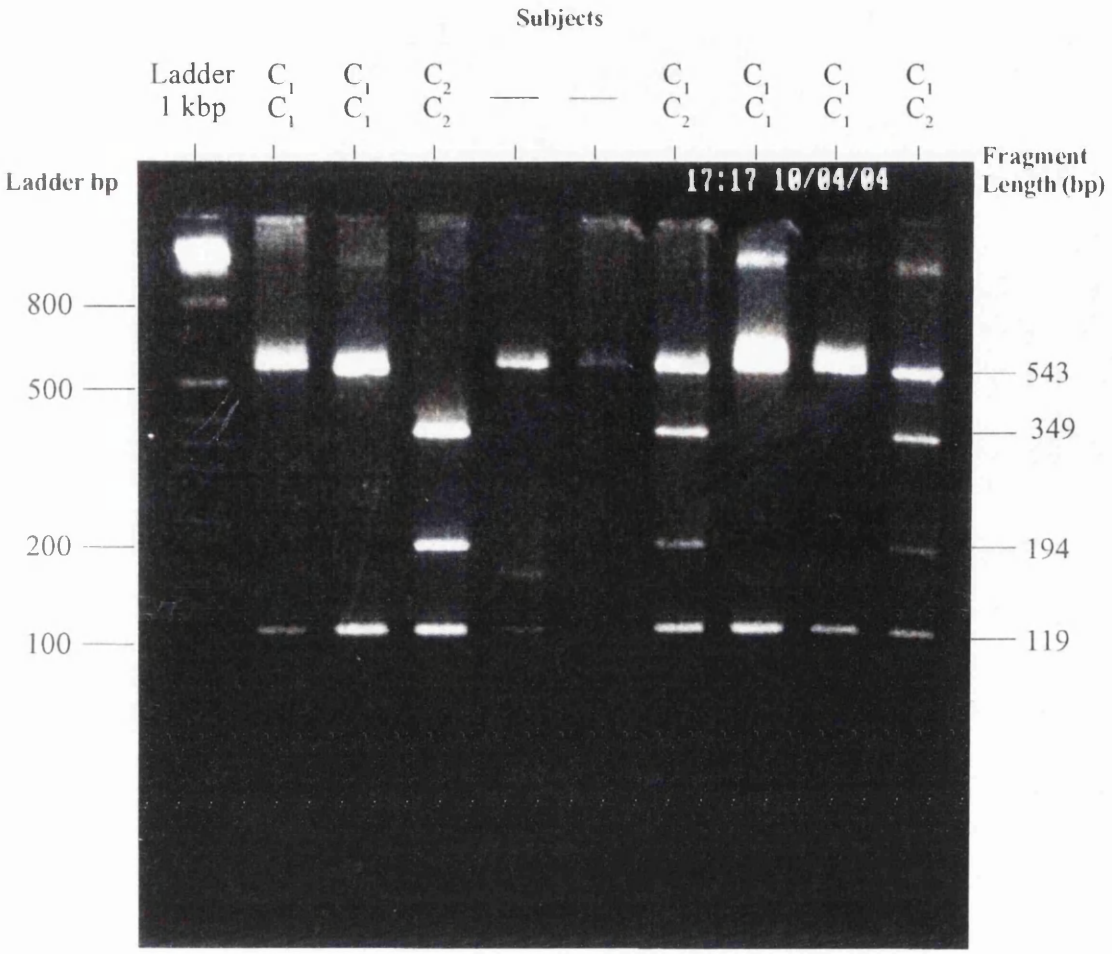


Figure 26 - Sample electrophoresis gel from nine subjects after digestion with Taq I

The amplified PCR product digested with Taq I results in two fragments of 543bp and 119bp when the C_1 allele is present, whereas digestion of the PCR product with the C_2 allele present revealed an additional Taq I cleavage site in the 543 bp fragment into 349bp and 194bp fragments. This explains the banding patterns in the above gel.

CHAPTER 3

RESULTS

3.1 CRANIOFACIAL RESULTS

The statistics protocol for analysis of the cephalometric data is described in section 2.3.3.3. Twenty of the 94 lateral cephalograms (21.3%), chosen at random, were retraced and redigitised. The reliability of measurement was checked by examining systematic and random errors associated with the replicate measurements.

3.1.1 Parental data

a) Systematic error.

The 95% confidence intervals for all but five variables, two angular, two linear and one area measurement, in the parental data contained zero (Table 14). These were the S-N to maxillary plane angle (s36), cranial base angle (N-S-Ba), ramus length (Cd-14), frontal chord (c1) and area of the cranial base (s41). Figures 27 (a) and (b) are examples of variables where significant bias was detected, for area of cranial base (s41) and cranial base angle (s48) respectively. These illustrate systematic error with variation above, (s48) and below, (s41) zero. Figure 27(c) illustrates an example (variable s50) where there was no systematic error in measurement i.e. distribution above and below zero was relatively evenly distributed. Recording of an important parameter in the context of this study variable s8, mandibular ramus length (Cd-14) was subject to a significant degree of systematic error ($p = 0.0014$). Figure 27(d) illustrates this bias in its measurement.

b) Random error

Figures 28(a), (b) and (c) are examples of angular (gonial angle, s11), linear (anterior face height, s24) and area (cranial area, c6) measurements which were shown to have a high intra-class correlation coefficient and therefore little random error. Seven of the 69 variables used in the parental study had intra-class correlation co-efficients less than 0.85, an arbitrary cut-off point that was adopted here (Table 14). Three of these (s55, s56, s57) were nasal measurements which were particularly unreliable in the parental sample because of poor definition on the radiographs. For this reason all nasal measurements were in fact discarded for the later multivariate analysis. Of the other four variables, only the mandibular ramus height (Cd-14) was important in the later analysis. Figure 28 (d) illustrates the

random error associated with this measurement. The reason for its poor random error rating (0.830) is thought to be mainly due to the poor reliability of locating Condylion (Cd).

Table 14 Cephalometric variables subject to systematic and/or random error in parental and control groups.

	Systematic error: 95% C.I. between 1st and 2nd measurement		Random error: Intra-class correlation co-efficient between 1st and 2nd measurement	
variable	Parents	Controls	Parents	Controls
s1 Me1-Me2	—	0·0039 .... 0·143*	—	—
s8 Cd-14	-3·745....1·006**	—	0·830	—
s12 Xi angle	—	-1·922.... -0·152*	—	—
s21 UIA	—	—	0·812	—
s26 Se1-PNS	—	—	—	0.745
s30 PNS-HOR	—	—	—	0.829
s34 NS-Max·plane	0·080.....0·509**	—	—	—
s40 N-S-Or-Po	—	—	—	0.847
s41 Area C. Base	0·029.....0·360*	—	—	—
s47 44-47	—	—	0·847	—
s48 N-S-Ba	-1·759....0·294**	—	—	—
s49 Cd-56	—	-1·053.... -0·305**	—	0.783
s51 FMN-N	—	—	—	0.807
s55 S-N-R	—	—	0·681	—
s56 N-R-A	—	—	0·806	—
s57 N-R-Or	—	—	0·742	—
c1 Fr Chord	-1·402....0·177*	—	—	—

significance of the differences for systematic error values.

* $p < 0.05$

** $p < 0.01$

— no significant difference.

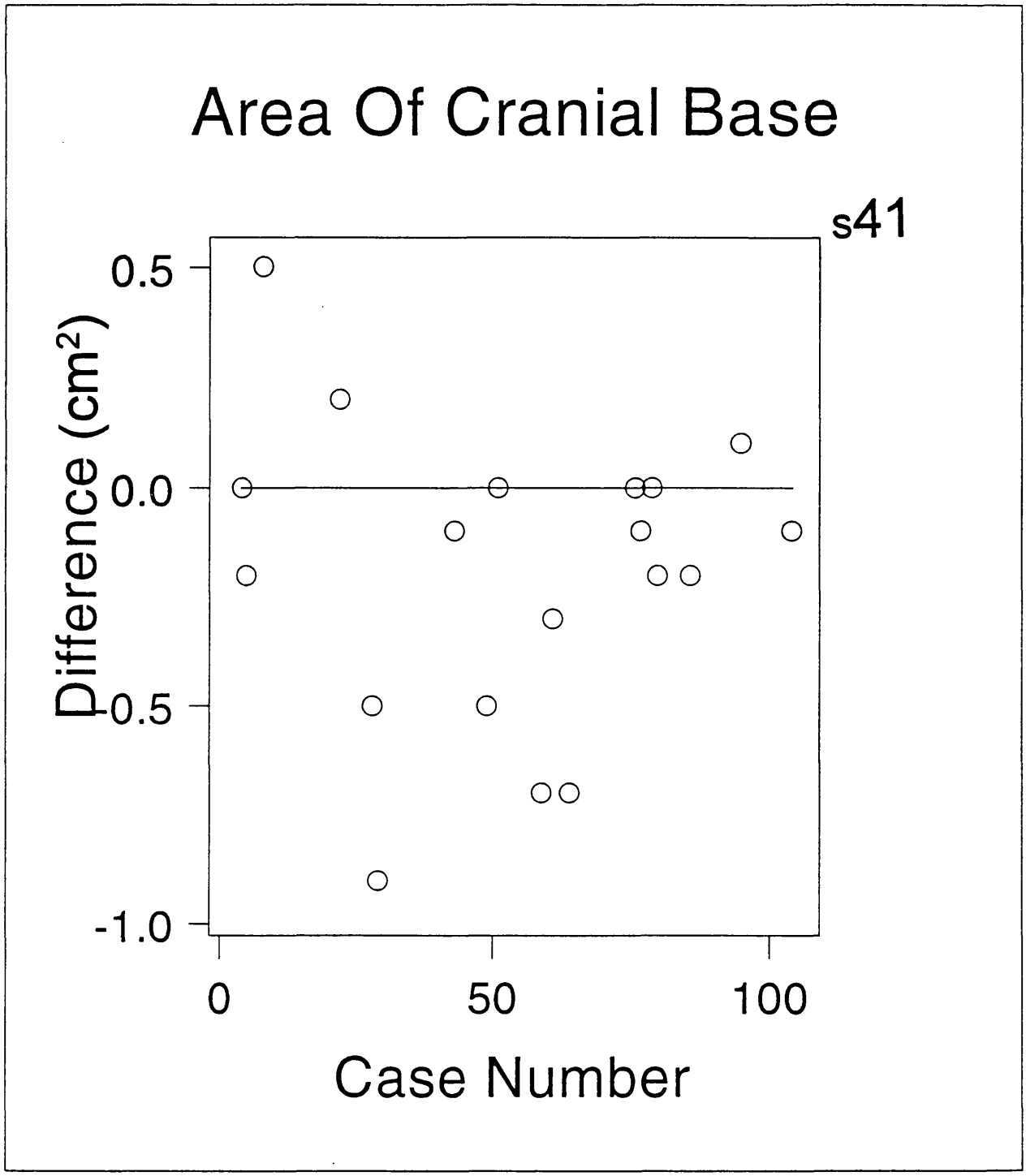


Figure 27 (a) Systematic error plot for repeated measurement of cranial base area showing disproportionate distribution below zero

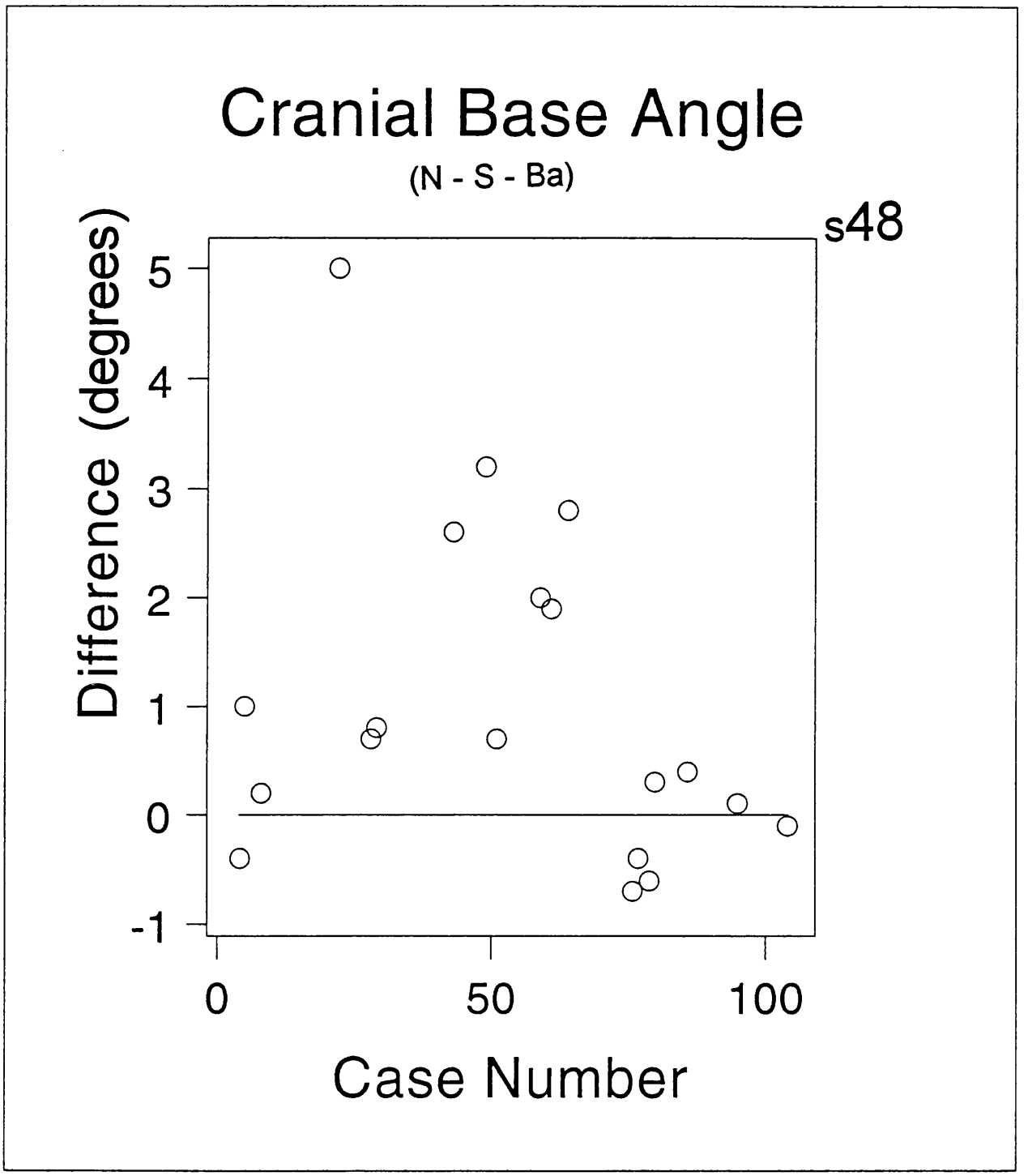


Figure 27 (b) Systematic error plot for repeated measurement of the cranial base angle showing disproportionate distribution above zero

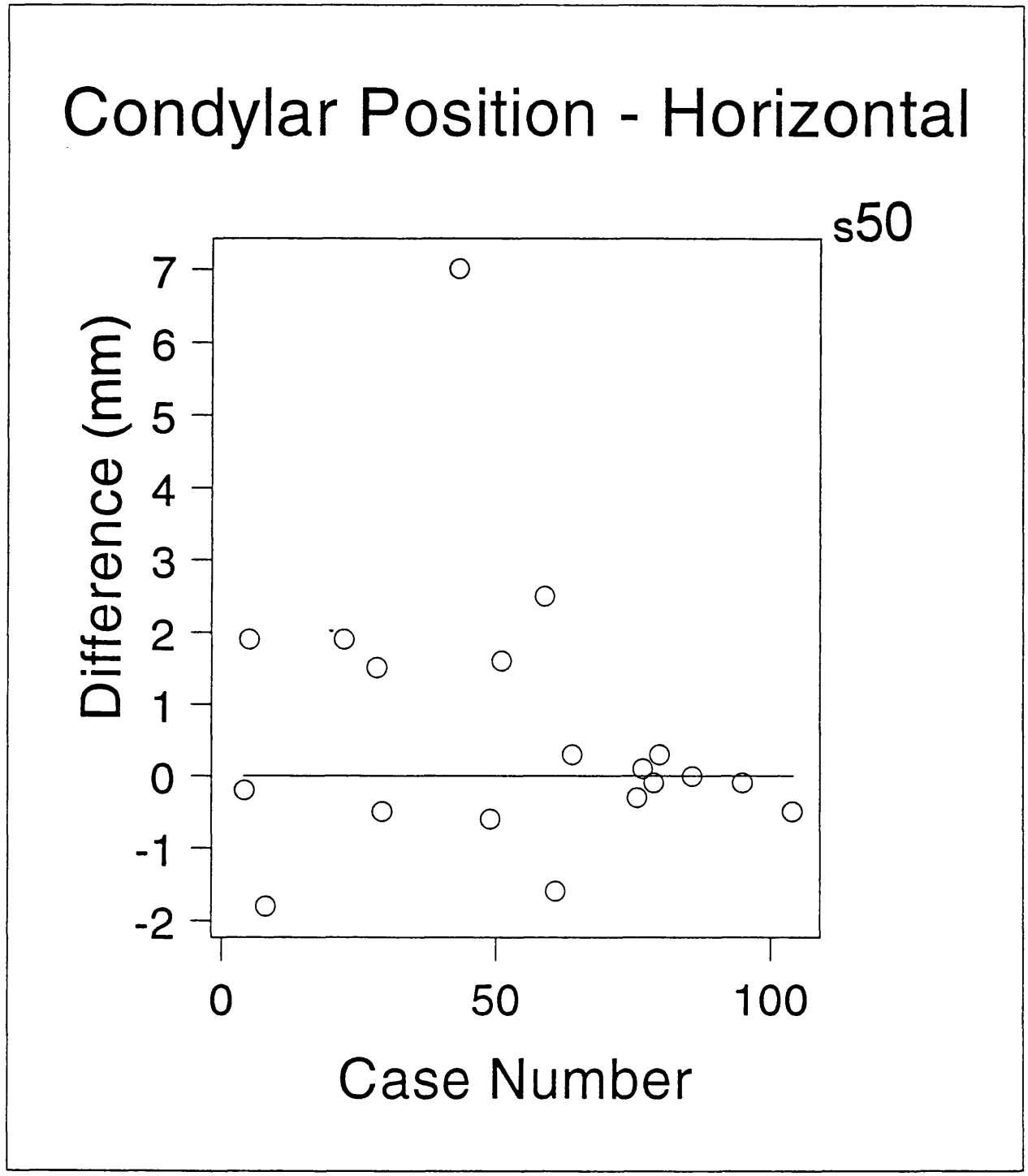


Figure 27 (c) Systematic error plot for repeated measurement of condylar position in the horizontal plane with fairly even distribution above and below zero

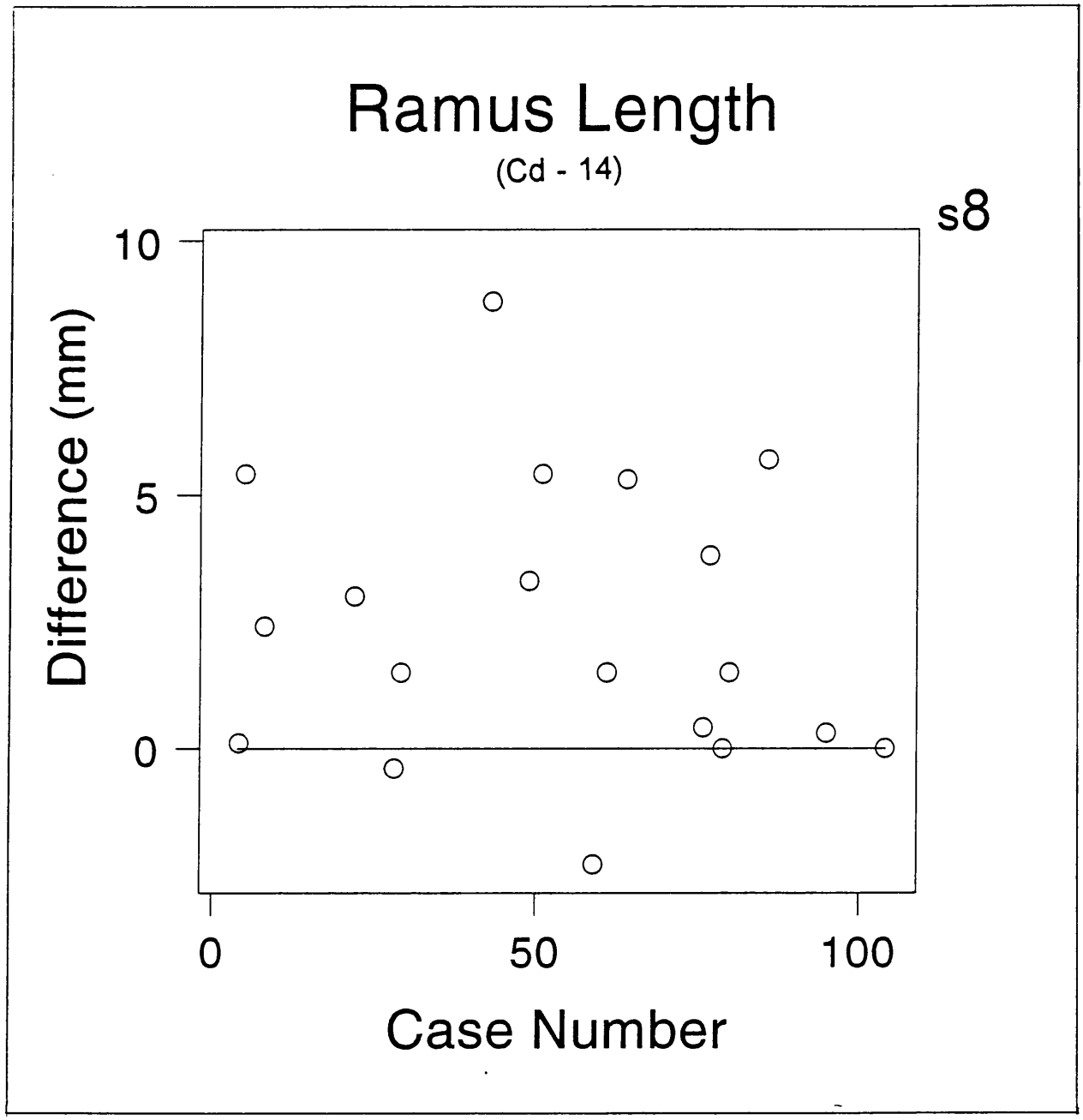


Figure 27 (d) Systematic error plot for repeated measurement of ramus length showing bias inherent in its measurement

To summarise:

i) Systematic error (bias) was checked by constructing 95% confidence intervals for the average difference between the first and second measurements. Out of the variables measured all but five of the confidence intervals contained zero, indicating no significant operator bias during measurement.

ii) Random Error was quantified by deriving intra-class correlation co-efficients of the variables. This indicated relatively poor reliability (intra-class correlation co-efficient of less than 0.85) for seven of the 69 variables. Some of these seven were discarded and only one, the mandibular ramus height (Cd-14) was found to be an important variable in the study.

c) Selection of variables for multivariate analysis

After careful vetting of the cephalometric variables measurement reliability, inter-variable correlation (see Table 7) and usefulness as described in section 2.3.3.3, 37 of the original 69 variables remained to be used for a multivariate analysis (see Table 8). Of these 37 variables three were earlier found to have some random error in measurement, four were subject to bias, and only one (Cd-14) had an element of both (see Figure 27(d) and Figure 28(d)).

d) Adjustment of data

All but eight of the variables used in the stepwise discriminant analysis showed gender effects, the male being invariably larger for area and linear measurements. Figure 29 (a), and (b) are superimposed mean tracings of male and female groups for the facial and cranial analyses respectively and these illustrate the size difference due to gender. None of these parental variables, however showed any age effect whatsoever. Adjustment for age was therefore not considered necessary but adjustment for gender was deemed important. All variables were adjusted to males in skeletal Class I. Since the mean value for males was greater than the mean value for females in 56 of the 67 variables the female data were adjusted by adding the difference in means between males and females to the female figure. After adjustment some figures needed to be log transformed which is a method of scaling down the data to give a better normal distribution while it remains valid for comparison.

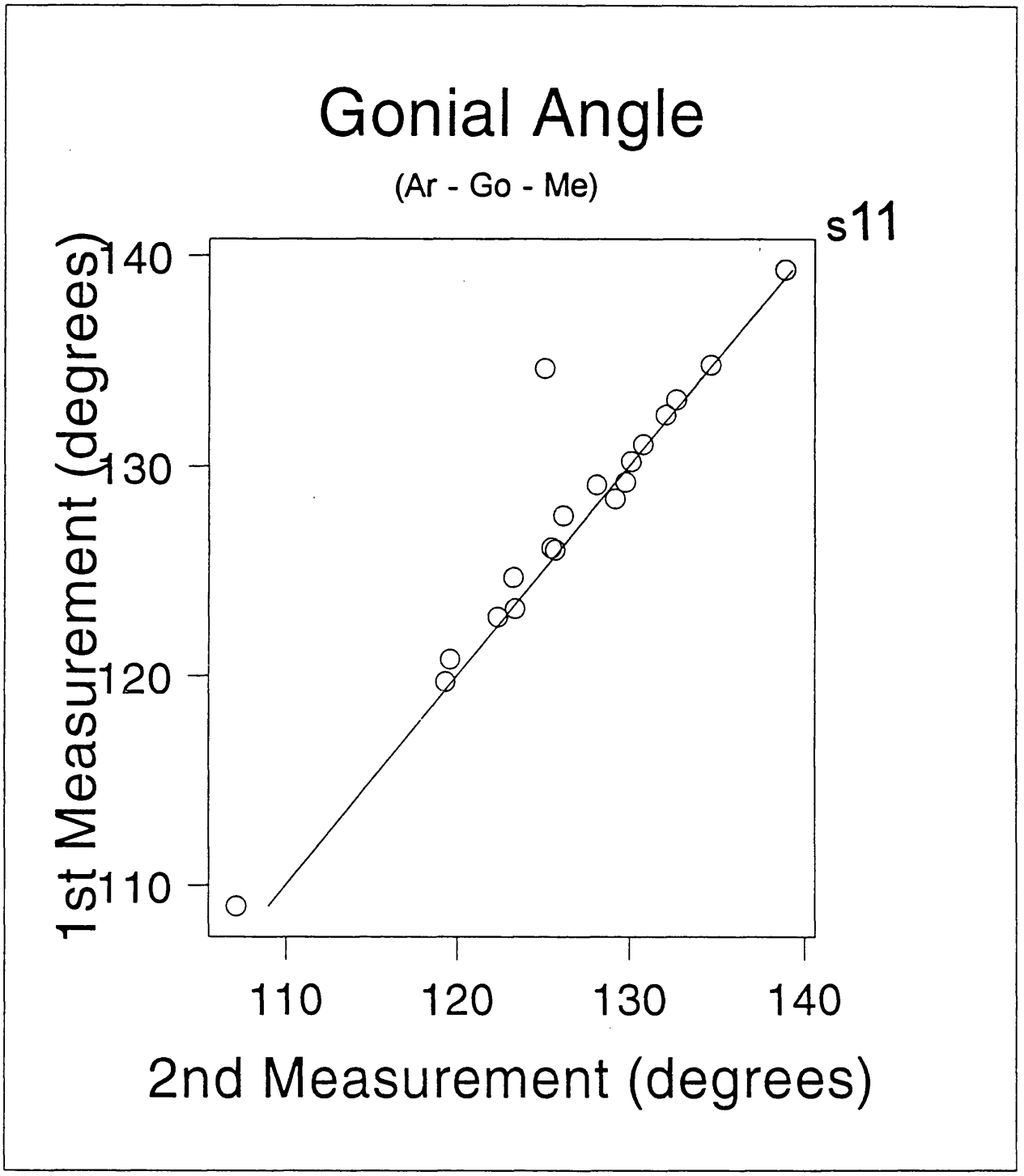


Figure 28 (a) Random error plot for repeated measurement of gonial angle showing little random error. Intra-class correlation coefficient = 0.939

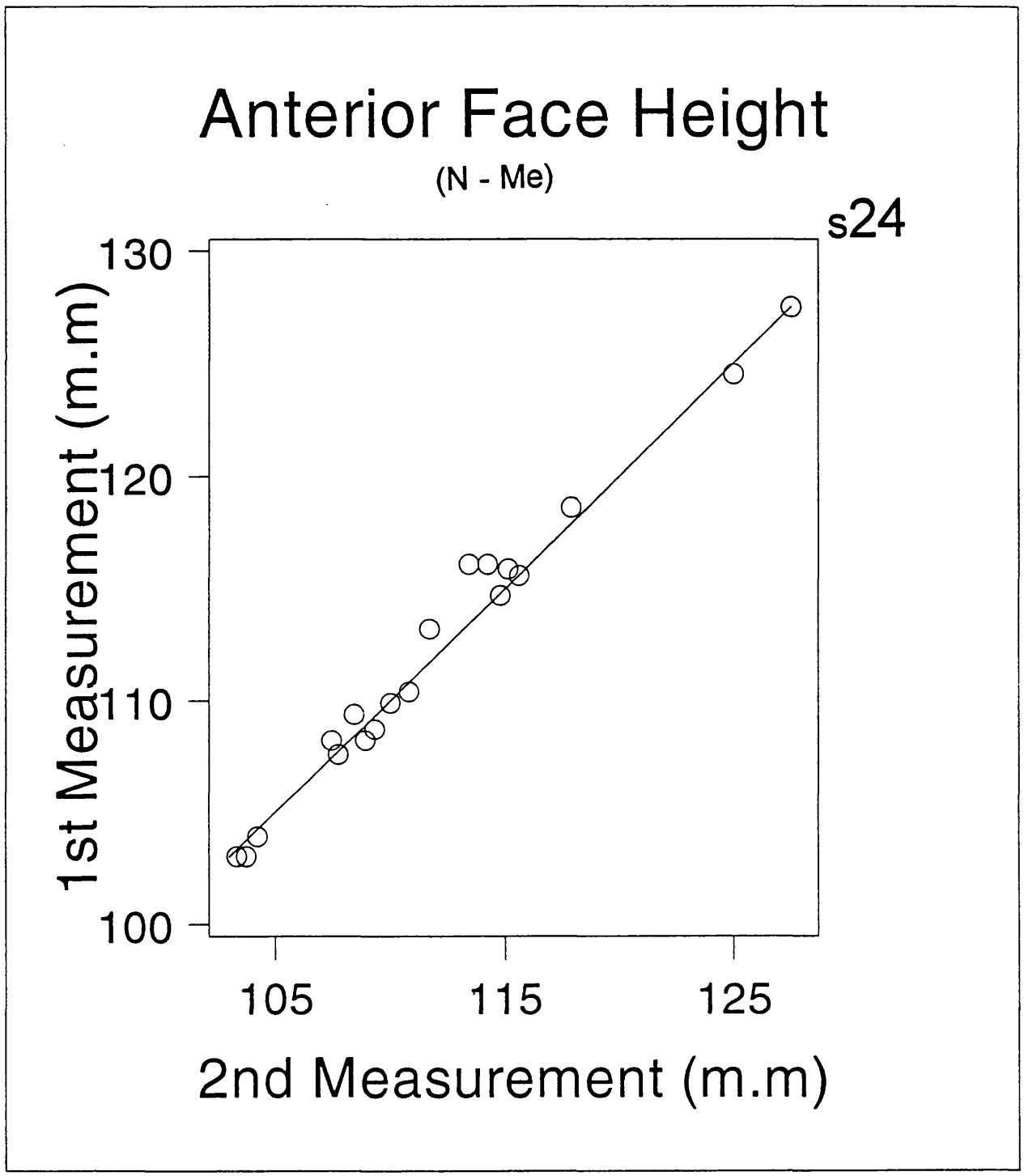


Figure 28 (b) Random error plot for repeated measurement of anterior face height showing minimal random error. Intra-class correlation coefficient = 0.989

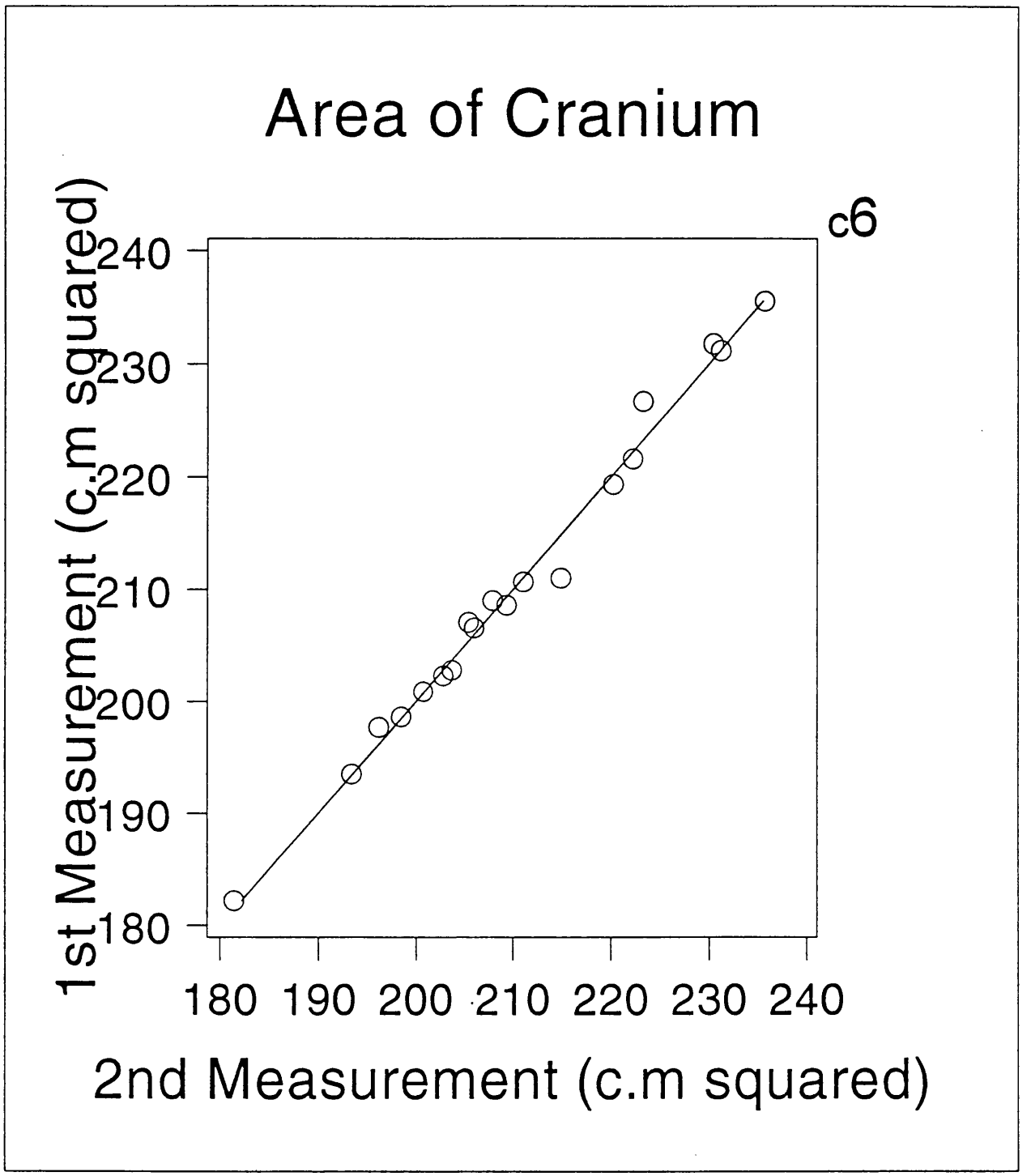


Figure 28 (c) Random error plot for repeated measurement of cranial area showing minimal random error. Intra-class correlation coefficient = 0.995

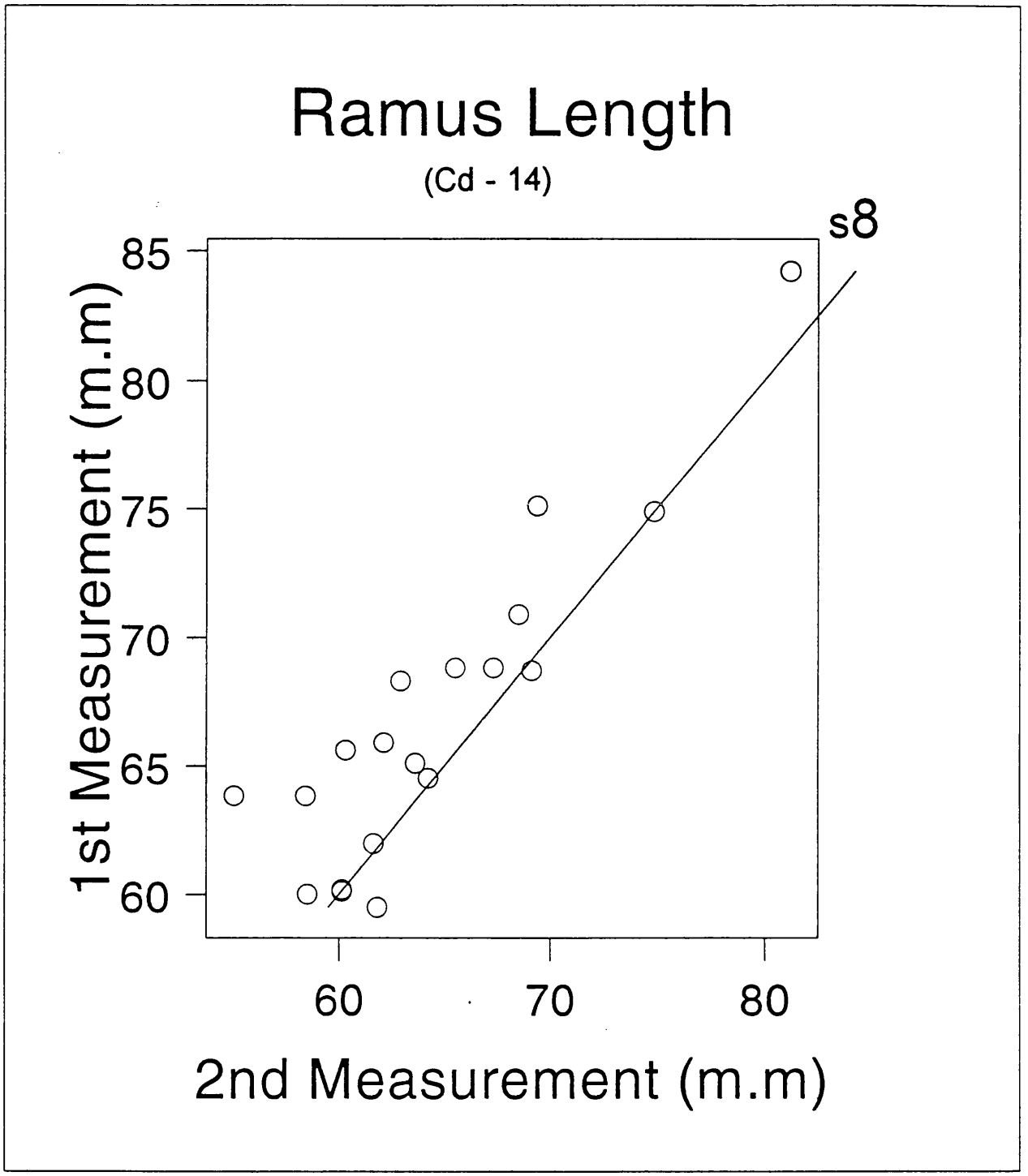


Figure 28 (d) Random error plot for repeated measurement of ramus length (Cd - 14) showing significant random error. Intra-class correlation coefficient = 0.830. Systematic error associated with measurement of this parameter is also apparent.

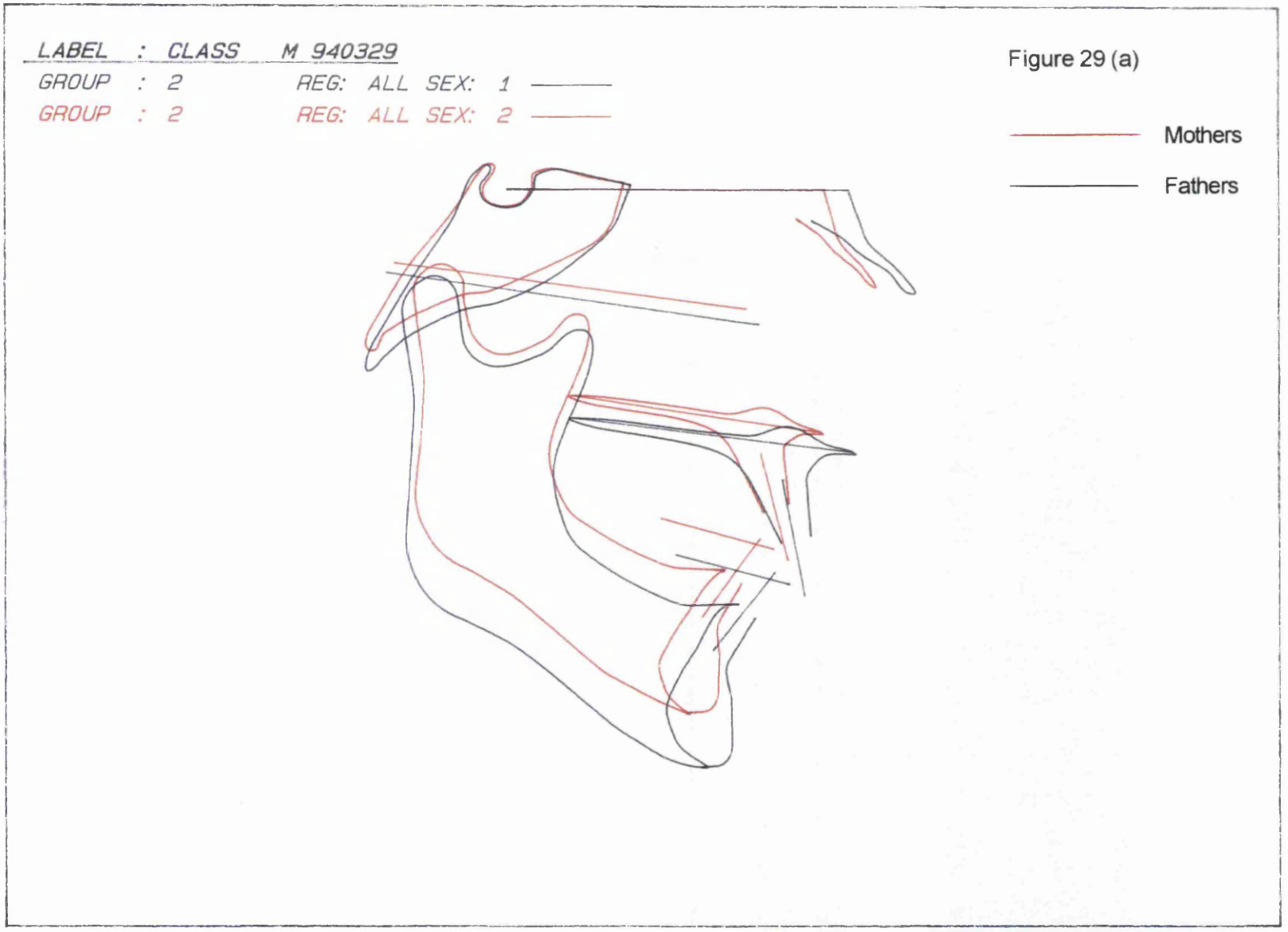


Figure 29 (a) Superimposition of mean facial plots for parental male and female groups

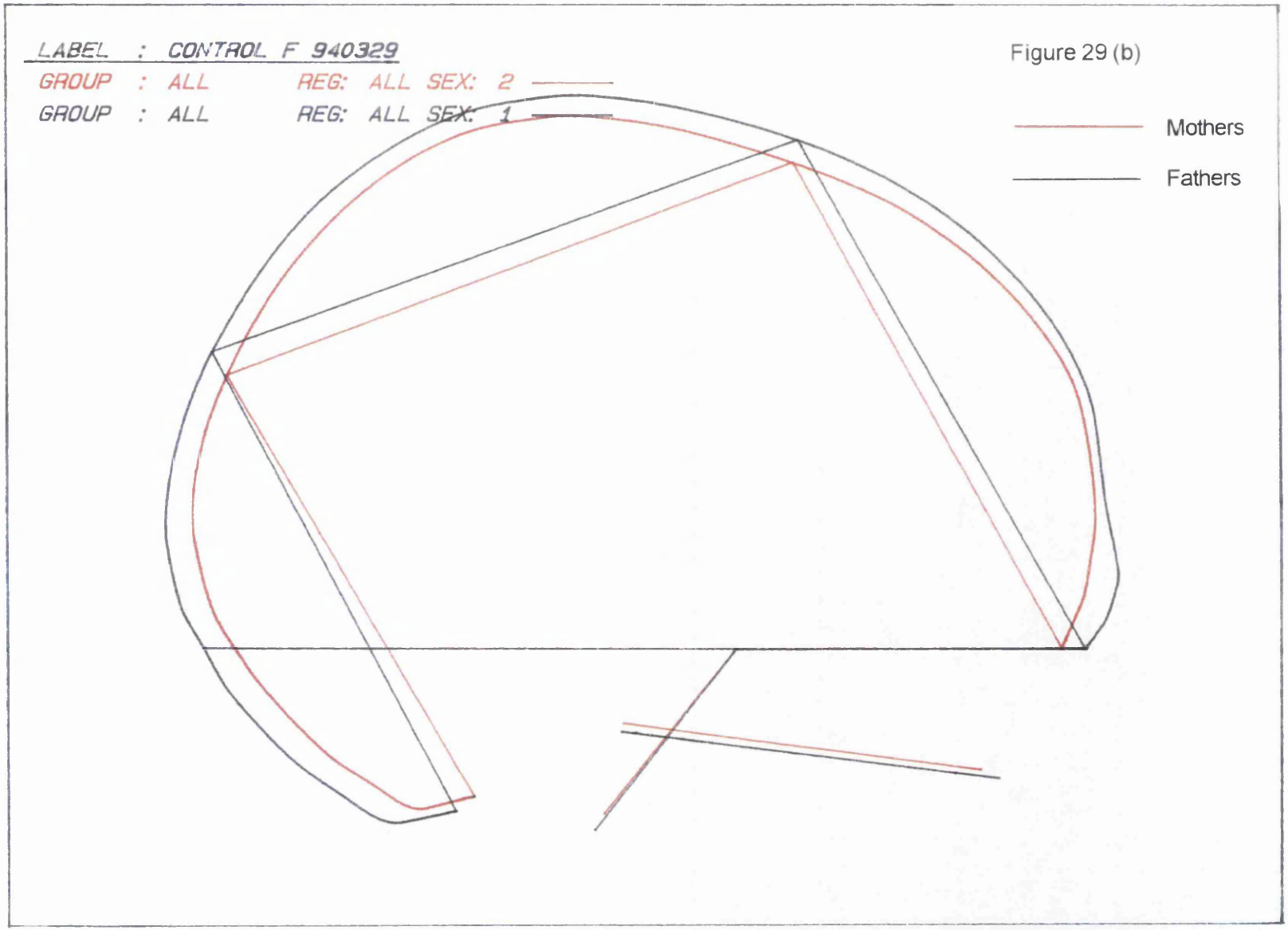


Figure 29 (b) Superimposition of mean cranial plots for male and female parental groups

e) Analysis of variance

The adjusted parental data were now tested for differences in the main cleft subsets, CL, CLP and CP. It is assumed that CL and CLP represent different degrees of severity of the same deformity, i.e., they share a similar aetiopathogenesis. It does not necessarily follow that the parents of children with either defect would present with similar cranial morphology. This could be tested by separating the parents of CL and CLP children for the purpose of a three-way comparison with the CP parents using a one-way analysis of variance (ANOVA). The significance of the differences in craniofacial measurements was tested by deriving 95% Tukey confidence intervals for the difference in pairwise means between groups.

The results of this test (Table 15) show that not a single craniofacial parameter of the 37 tested differed significantly between the CL and CLP groups, and only four emerged as being statistically significantly different between CP and CLP. All of these being larger for the CP parental group (Figure 30(a) and 30(b)). These were the cranial area (c6), mandibular area (s2), maxillary-mandibular planes angle (s35) and mandibular ramus length (Cd-14). It should, however be noted that three of the above results are borderline at the 5% level of significance, and since there are 37 tests being carried out, it is to be expected that a small number of the tests will be significant at this level by chance alone. The difference in the fourth parameter, the mandibular ramus length (Cd-14), reached a higher level of statistical significance, ($p = 0.013$). However, the accuracy of this parameter is in question since it suffers from bias and a low intra-class correlation (0.830).

It can therefore be concluded that no statistically significant differences exist between parents of children with CL and CLP, and therefore the pooling of these parents into a combined CL(P) group can be justified. Also analysis of variance using mean values for the different cleft types reveals little if any difference between the parents of isolated cleft palate children and those whose children have cleft lip with or without cleft palate. This study also highlights the problem with measurement of mandibular ramus length (Cd-14). The greater part of the

variation in repeated measurement of this parameter thought to be due to the uncertainty of identification of condylion (Cd). In the majority of lateral skull radiographs this landmark is somewhat obscured due to superimposition by the petrous temporal bone.

Table 15 Comparison of CP/CL/CLP parental craniofacial morphology using a one way analysis of variance.

Variable	Group Means	95% Tukey Intervals	p-Value for ANOVA
Mandibular	1) 31·126	1-2: -1·665 ....2·638	0·049
area (s2)	2) 30·640	1-3: 0·005 ....3·438	
	3) 29·380	2-3: -0·991 ....3·431	
Ramus	1) 68·698	1-2: -0·169 ....6·454	0·013
length (s8)	2) 65·555	1-3: 0·379 ....5·586	
	3) 65·716	2-3: -3·502 ....3·181	
MMPA	1) 3·0698	1-2: -0·166 ....0·194	0·047
(s35)	2) 3·0056	1-3: -0·274 ....0·008	
	3) 3·2028	2-3: -0·329 ....0·034	
Cranial area	1) 5·4101	1-2: -0·004 ....0·085	0·049
(c6)	2) 5·3699	1-3: -0·006 ....0·064	
	3) 5·3808	2-3: -0·056 ....0·034	

Key; 1 = CP
2 = CL
3 = CLP

f) Stepwise discriminant analysis

The purpose of a stepwise discriminant analysis was to determine which variables discriminate best between CL(P) and CP parents. Before the Stepwise Discriminant Analysis was undertaken t-tests between CP and CL(P) were carried out on each variable (Table 16). Five of the craniofacial variables were found to be significantly different for CP parents and CL(P) parents ($p < 0.05$). These were mandibular area (s2), ramus length (Cd-14), total mandibular length (Cd-Gn), area

of the cranium (c6) and length of the occipital chord (c3). Again all of these measurements were larger for cleft palate (CP) parents (Figure 30 (a) and (b)).

Stepwise Discriminant Analysis to discriminate between CP and CL(P) was then undertaken for three different cases:

1. Each parent as a separate subject.
2. Couples combined as one case, and
3. Separate analyses for fathers and mothers.

This analysis was carried out assuming prior probabilities of one in two. This was to reflect that in prevalence studies, the ratio of CL(P) to CP in the West of Scotland is 50:50, whereas in the rest of Britain surveys indicate that the ratio is approximately 70:30.

The poorest overall discrimination was with the couples combined as one case when CP and CL(P) parents were correctly distinguished in 69.2% and 60% of cases respectively. Analysis of each parent as a separate subject discriminated marginally better with 71.4% of CP and 62.5% CL(P) parents being correctly classified. Separating the sexes improved discrimination to 75% and 80% for CP and CL(P) respectively for mothers when ramus height (Cd-14) and cranial height (V-Ba) were entered; and for fathers 68.4% of CP and 65.2% of CL(P) were classified correctly with the entry of ramus height (Cd-14) alone .

In summary few parameters appear to be significantly different in the stepwise analysis of the entire group as a whole or of the couples as one case. Analysis of mothers and fathers separately however, indicated that ramus height (Cd-14) and cranial height (V-Ba) together in mothers of cleft children were shown to be a reliable distinguishing factor between CL(P) and CP.

Table 16 Comparison of cephalometric values between CL(P) and CP groups using two sample t-test. (adjusted data).

	CP n=35		CL(P) n=48			
#Variable	Mean	S.D.	Mean	S.D.	95% C.I.	p-value
s1	3·17	0·44	3·141	0·38	-0·167 ....0·203	0·84
s2	31·13	3·09	29·77	2·82	0·03 ....2·67	0·045*
s3	79·49	5·40	78·64	3·88	-1·30 ....2·99	0·43
s7	116·02	5·72	113·67	3·75	0·14 ....4·58	0·038*
s8	68·70	4·57	65·67	4·38	1·04 ....5·02	0·0033*
s11	126·49	6·16	127·47	4·85	-3·4 ....1·59	0·46
s14	3·85	0·72	3·62	0·66	-0·08 ....0·54	0·14
s15	51·14	3·57	51·5	3·38	-1·91 ....1·18	0·64
s18	3·31	0·07	3·29	0·10	-0·023 ....0·053	0·44
s24	118·83	6·52	117·22	5·51	-1·1 ....4·33	0·24
s25	53·31	3·23	52·08	2·95	-0·15 ....2·61	0·08
s26	47·14	3·08	46·31	2·92	-0·5 ....2·17	0·22
s27	4·83	0·04	4·80	0·03	-0·002 ....0·036	0·72
s30	27·73	2·29	26·93	2·53	-0·26 ....1·86	0·14
s31	3·79	0·04	3·79	0·05	-0·015 ....0·027	0·55
s32	80·99	3·46	79·97	3·47	-0·52 ....2·55	0·19
s33	78·11	3·86	77·43	3·26	-0·93 ....2·28	0·40
s35	3·07	0·25	33·16	0·25	-0·197 ....0·02	0·12
s39	56·92	4·12	56·17	3·61	-0·98 ....2·49	0·39
s40	8·43	3·16	7·61	3·57	-0·6 ....6·30	0·27
s41	10·02	1·05	9·83	0·94	-0·26 ....0·63	0·42
s42	67·99	3·07	67·60	3·02	-0·97 ....1·73	0·57
s44	44·70	2·09	45·19	2·52	-1·50 ....0·52	0·33
s47	10·23	1·31	10·29	0·89	-0·57 ....0·45	0·82
s48	126·16	5·43	128·00	5·36	-4·24 ....0·54	0·13
s49	12·40	3·00	13·19	2·62	-2·06 ....0·47	0·21
s50	19·13	2·52	19·47	2·53	-1·46 ....0·78	0·55
s51	-0·007	0·22	-0·015	0·22	-0·09 ....0·11	0·88

refer to Table 7(a)

Table 16 (cont.) Comparison of cephalometric values between CL(P) and CP groups.

	CP n=35		CL(P) n=48			
#Variable	Mean	S.D.	Mean	S.D.	95% C.I.	p-value
c1	112·01	5·32	111·10	5·53	-1·49 ....3·30	0·45
c2	122·77	4·81	121·81	4·83	-1·18 ....3·09	0·37
c3	103·85	6·04	101·15	5·84	0·1 ....5·34	0·045*
c4	4·97	0·04	4·96	0·036	-0·01 ....0·02	0·41
c5	188·30	6·38	185·81	5·39	-0·2 ....5·15	0·066
c6	5·41	0·06	5·38	0·06	0·006....0·06	0·018*
c7	25·18	2·33	25·37	2·87	-1·32 ....0·95	0·75
c8	26·90	2·41	27·01	2·57	-1·22 ....0·98	0·83
c9/c10	33·29	3·68	31·58	4·71	-0·12 ....3·55	0·67

* significant differences at $p < 0·05$

refer to Table 7(b)

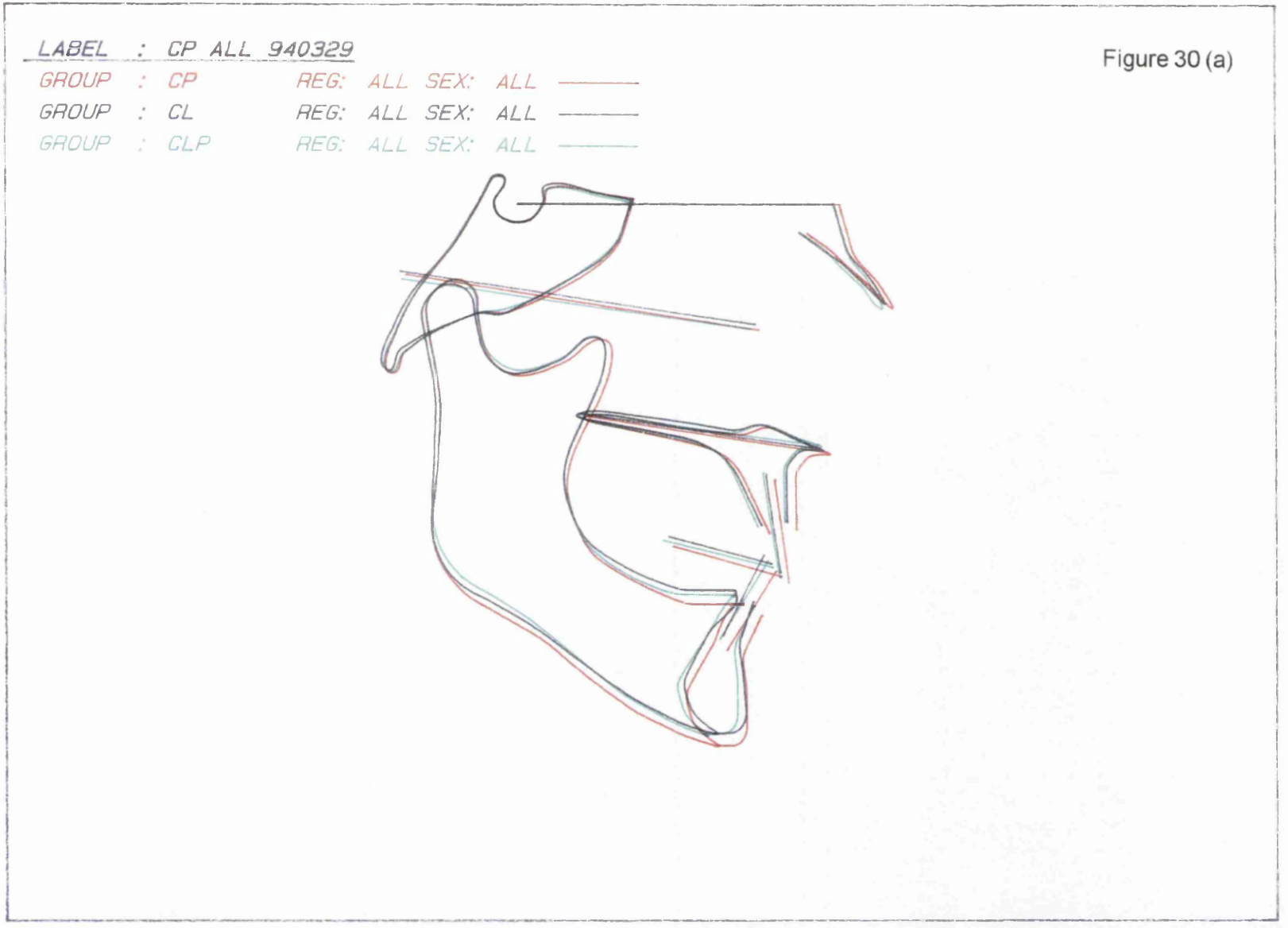


Figure 30 (a) Superimposition of mean facial plots for CP, CL and CLP parents

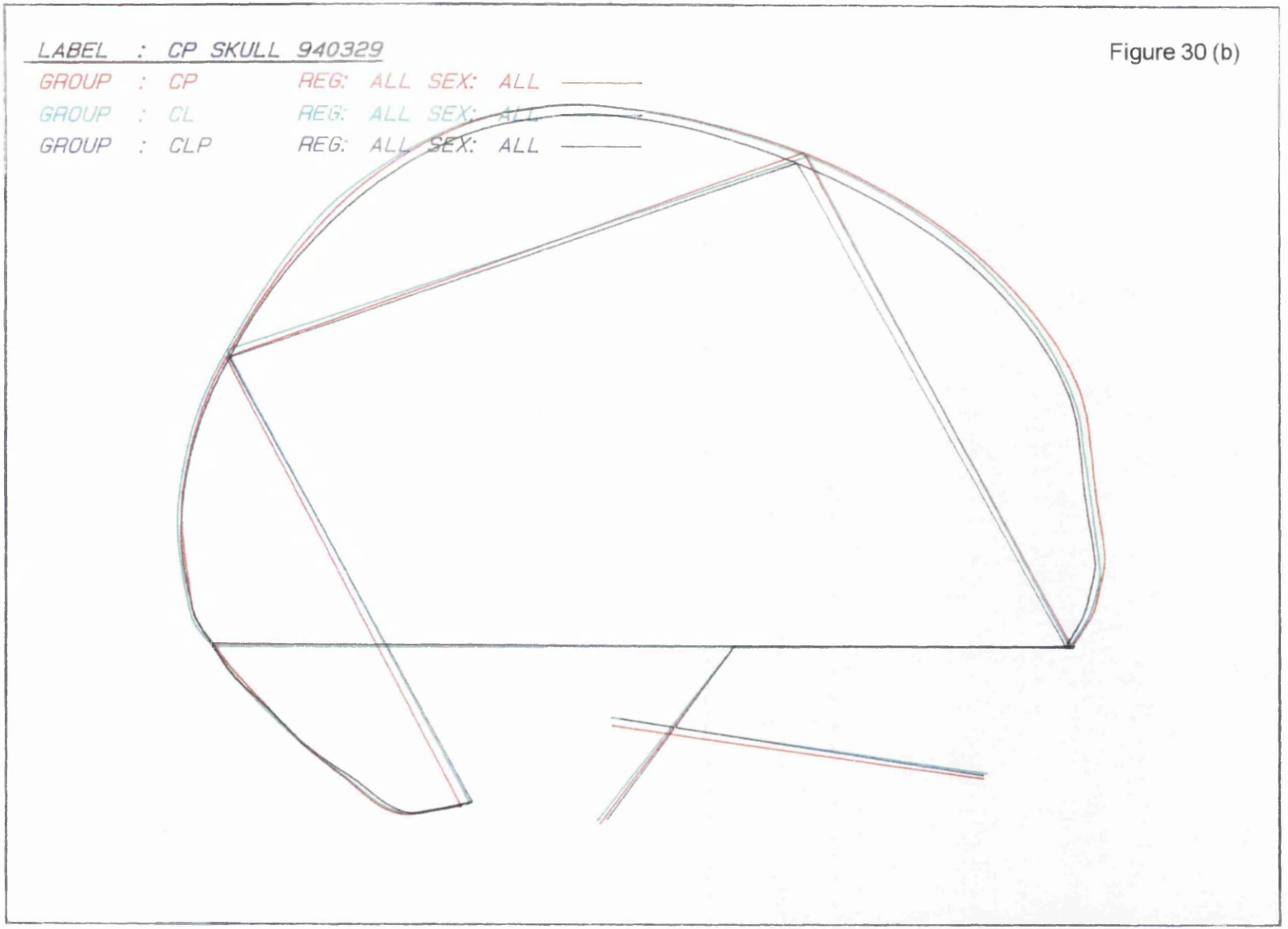


Figure 30 (b) Superimposition of mean cranial plots for CP, CL and CLP parents

3.1.2 Analysis of control data

The sample of 95 control radiographs, chosen as described above, were analysed in a similar manner to the parental data. Twenty cases (21%) were randomly selected for re-digitising as before and an error analysis carried out on all 67 craniofacial variables using the same techniques that were used in parents, i.e. t-tests of the paired differences to check for bias and intra-class correlation co-efficients to evaluate random error. Only three of the variables symphyseal area (s1), Xi angle (Cd,Xi,pm) and the vertical position of the condyle (s49) showed a significant bias and five of the variables had an intra-class correlation co-efficient of less than 0.85 (see Table 14). The sets of variables found to exhibit relatively large errors in the parental and in the control data are mutually exclusive, and the vast majority of measurements showed a high degree of reliability. As always, we must accept the probability that some suggestion of bias will appear by chance, even when there really is none.

In preparation for comparison of parental and control groups the same 37 variables that were carefully chosen for the parental data, were picked out from the control data. Comparison of the skeletal class in both parental and control groups shows that differences in the relative proportions of each do exist (see Table 4). All of the 37 variables were then checked for the effects of age, gender and skeletal class. It was noted that the age range of the control was somewhat lower than that of the parents (see Figure 10). The vast majority of the control were in their early 20's which contrasted with the parents where the lowest age was 24.5 years and the mean age 37.6 years. It was possible therefore, that there could be differences between the groups, due to age effects, if growth continues into the third decade and beyond. The variables were plotted against age and labelled by gender. Three variables were found to be affected by a statistically significant gender/age interaction (Table 17). The interpretation of these is that in females the upper face height Figure 31 (a), cross sectional area of the cranial base Figure 31 (b),and area of the nasal bones Figure 31 (c), all appeared to get larger with age, even after 20 years old. Referring to the work done by Behrents (1985) has shown that there are subtle changes in the craniofacial skeleton beyond the age of

Table 17 Control variables showing a correlation with sex, age or skeletal class.

#Variable	Sex	Age	Skeletal Class	Interaction (s)
s1	*			
s2	*			
s3	*		*	
s7	*		*	
s8	*			
s14	*			
s15	*		*	
s18	*			
s24	*			
s25	*	*		sex*age
s26	*			
s27	*		*	
s30	*			
s31	*			
s32			*	
s33			*	
s41	*	*		sex*age
s42	*			
s44	*			
s47	*			
s49	*			
s50	*			
s51	*	*		sex*age
c1	*			
c2	*			
c3	*			
c4	*			
c5	*		*	sex*s. class
c6	*			
c7	*			
c9/c10	*			

refer to Table 7(a) and (b)

Figure 31: (a) - (c)

Sex/Age Interaction Plots for the Control Group

This was found to be significant for three craniofacial parameters

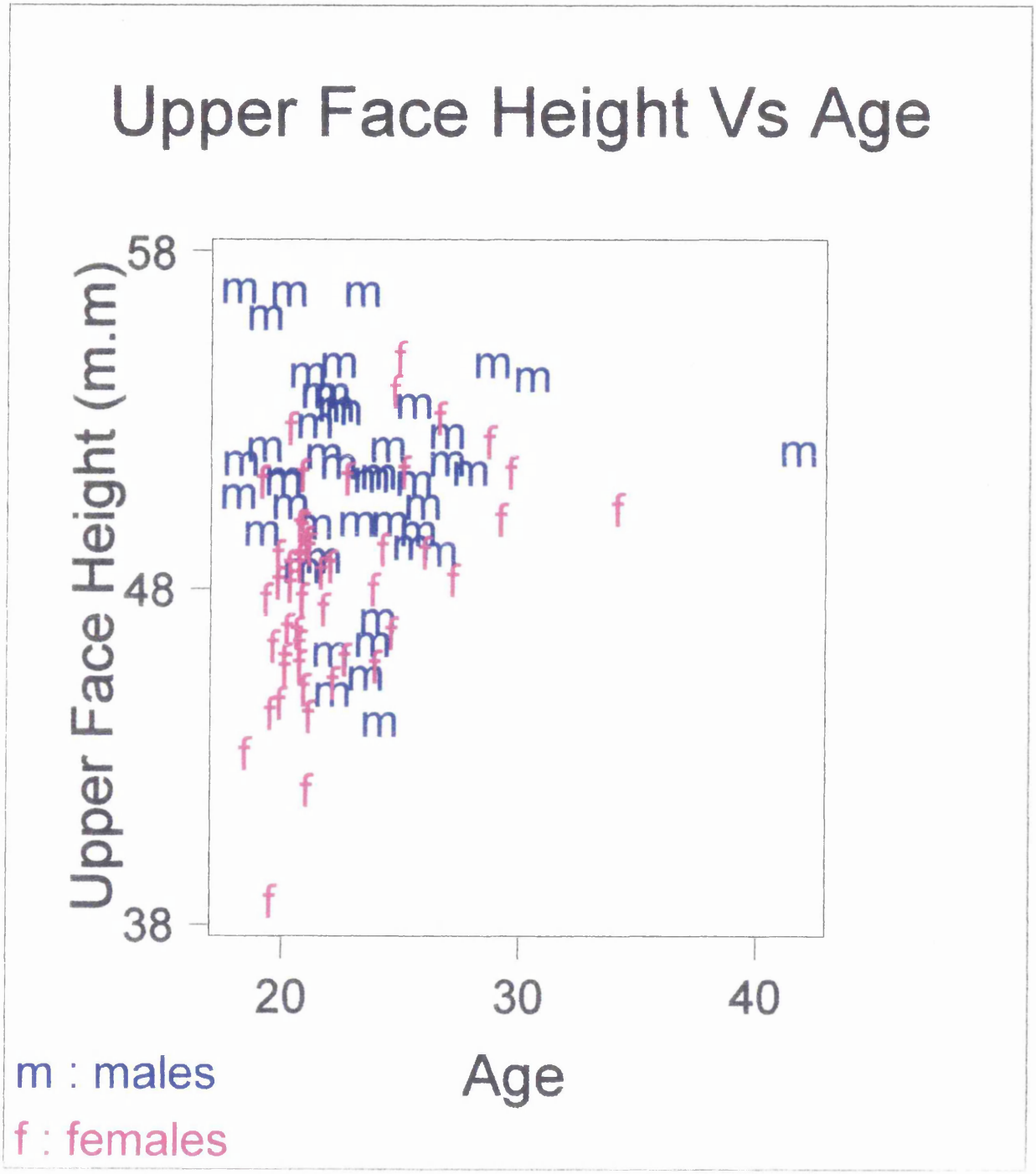


Figure 31 (a)

Variation in the upper face height (N-ANS) with age for male and female control subjects

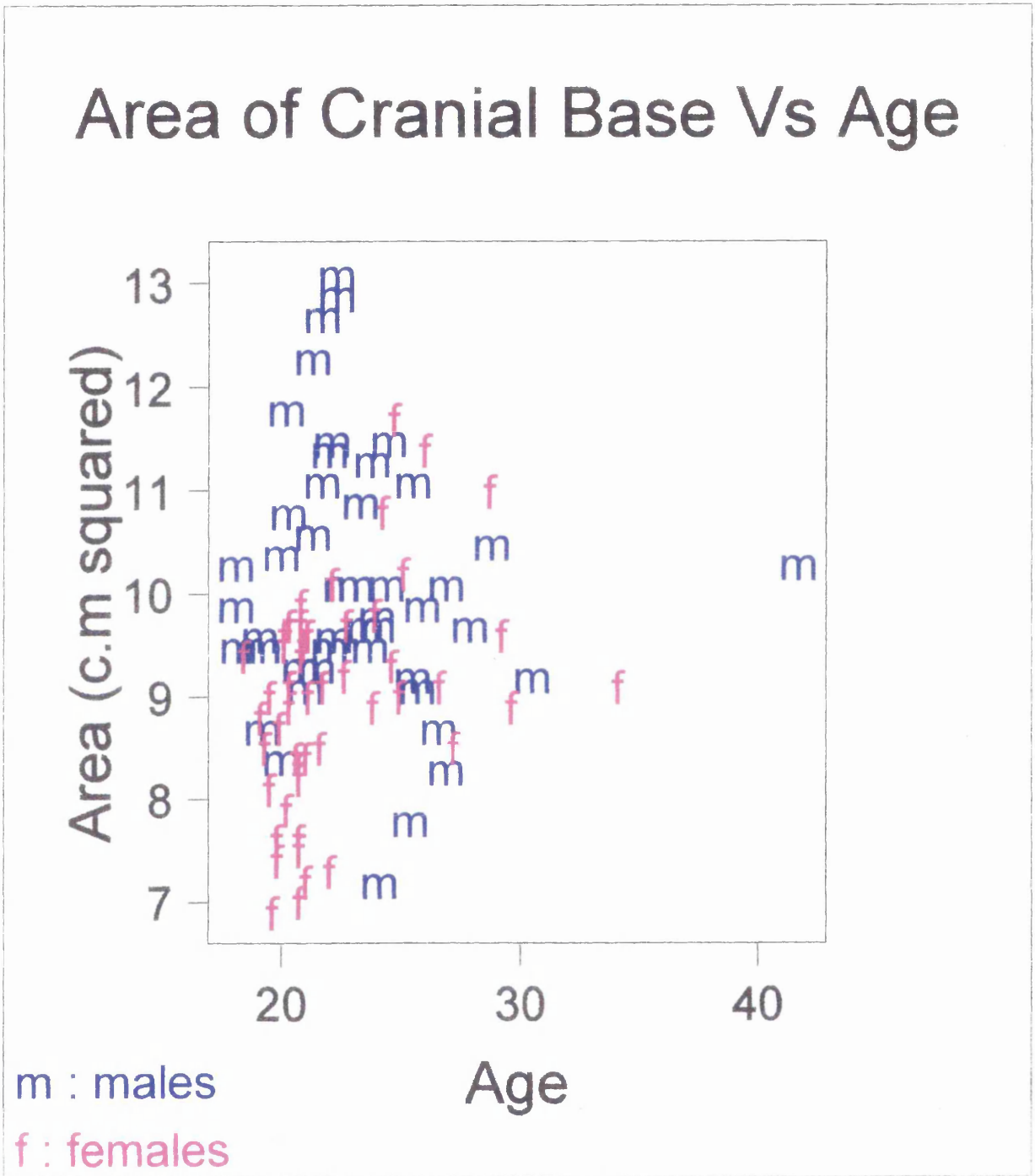


Figure 31 (b)

Variation in the area of the cranial base (s41) with age for male and female control subjects

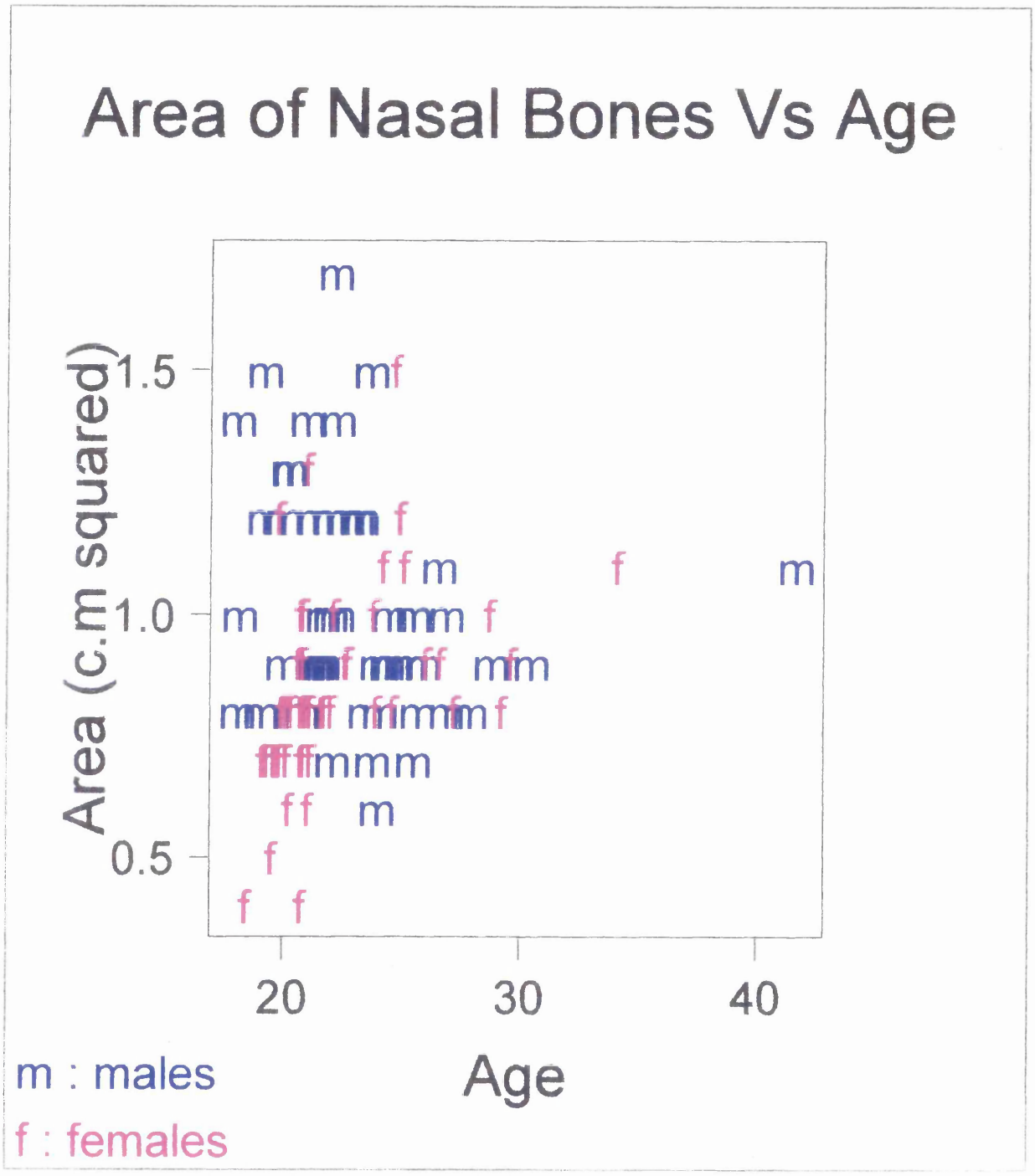


Figure 31 (c)

Variation in the area of the nasal bones (s51) with age for male and female control subjects

twenty years and subtle changes in the cranial base, upper face height and nasal bones are mentioned in his work. The magnitude of these later growth changes is, however, clinically insignificant and for the purposes of this study will be discounted. For the three variables in which an age effect was identified, these were minimal and to reduce the complexity of further analysis, it was decided that any age effect would be ignored. Nakasima and Ichinose (1983) encountered a very similar problem in their study on parental craniofacial morphology. Their sample of parental subjects whose ages ranged between 23.7 and 35.2 years were compared to a control group whose mean age was 23.2 years. Testing each measurement for an age effect using correlation coefficients, revealed none and they analysed their results without reference to age.

ii) Adjustment of the Control data

Prior to carrying out any multivariate analysis, it was necessary, after identification of the gender and skeletal class effects to adjust the variables accordingly. As with the parental data the control data was adjusted to males in skeletal class I. All but eight of the parameters were apparently subject to a gender effect. Age seemed to affect the average value of three of the variables as a gender/age interaction while there was an apparent skeletal class effect for seven variables. Table 4 (section 2.1.2) indicates the varying proportions of skeletal class within both the parental and control groups. Adjustment was done by adding the difference in the means between the different sexes, or skeletal classes to the lower value. Therefore to adjust a linear parameter for gender, the difference in the means of the male and female values was added to the female value. To quote an example, for variable s3 (mandibular area) which had to be adjusted for effects of both gender and skeletal class, the difference in the means between males and females (7.167) was added to the females and then the differences in the means between skeletal class I and II (1.188) and skeletal class I and III (-3.795) were added to all people in skeletal class II and III respectively.

The idea of producing adjusted data was to eliminate gender and skeletal class effects from the data so that;

a) by allowing the male and female data to be pooled, this effectively doubled the numbers in each group facilitating statistical analysis,

b) a direct comparison could be made of the parental and control groups uncomplicated by possible biases introduced by skeletal class and gender.

To demonstrate whether there is a distinct maternal or paternal effect during a later analysis of the parental data, then scrutiny of the data unadjusted for gender will be necessary.

3.1.3 Comparison of parental and control data.

Comparison of the parental and control data was the primary objective of this cephalometric study. Three distinctly different statistical tests were used to elucidate any differences, a Mahalanobis distance analysis, a two-sample t-test and a stepwise discriminant analysis.

I) Mahalanobis distances

In this study the Mahalanobis distances of the controls and parents were derived with respect to the mean vector and covariance matrix of the control sample.

i) Labelled by group: the plots indicated that there was a large difference between parents and controls (mean of the parents = 127.64 and mean of the controls = 36.611). Figure 32 (a) is a dotplot distribution to illustrate the distinctive differences between parents and control.

ii) Labelled by gender: there was little difference between males and females among the controls, as expected since the data had been adjusted for gender differences. In the parents, though, the females had a greater Mahalanobis distance on average (the mean of the females = 139.66 and mean of the males = 115.90 (Figure 33 (a) and (b)) are dotplot and box and whisker plots to illustrate this male/female subdivision and comparison.

iii) Labelled by skeletal class: there was little difference among the controls (again due to adjustment), but in the parents skeletal class II is significantly greater than I or III which are almost equal (mean of skeletal class I = 122.56, mean of skeletal class II = 142.9 and mean of skeletal class III = 120.91) (Figure 34(a) and (b)).

These results indicate that there are definite differences between the craniofacial morphology of the parental group and that of the controls. They also show that mothers differed more from the female control than did fathers from the male control with regard to craniofacial morphology; and skeletal class II parents had a greater Mahalanobis distance than those in the skeletal class I and III categories and therefore their craniofacial form deviates more from the control group than other parents.

In order to identify which parameters differ most between the maternal and paternal groups relative to their respective controls, each variable was taken individually and the difference between the two groups analysed using a two sample t-test.

II) Two sample t-tests

Because of the gender difference in craniofacial morphology highlighted by the Mahalanobis distance analysis, the t-tests were derived for males and females separately on data adjusted only for skeletal class effect. The results of this analysis is shown for each of the 37 variables in Table 18 which records the 95% confidence intervals and p-values. This highlights the parameters which show a statistically significant difference between either of the parental and their respective control groups. Box and whisker plots were also produced for each of the 37 variables, each plot illustrating the male/female comparison and subdividing the different cleft groups within each gender. In effect this allows a simultaneous comparison of differences between cleft types within and between the maternal and paternal groups, and between each parental group and their relative control (see appendix C). Histograms showing the particular parameters which differed between the mothers and fathers and their respective controls are included in the results. The following summarises these differences:

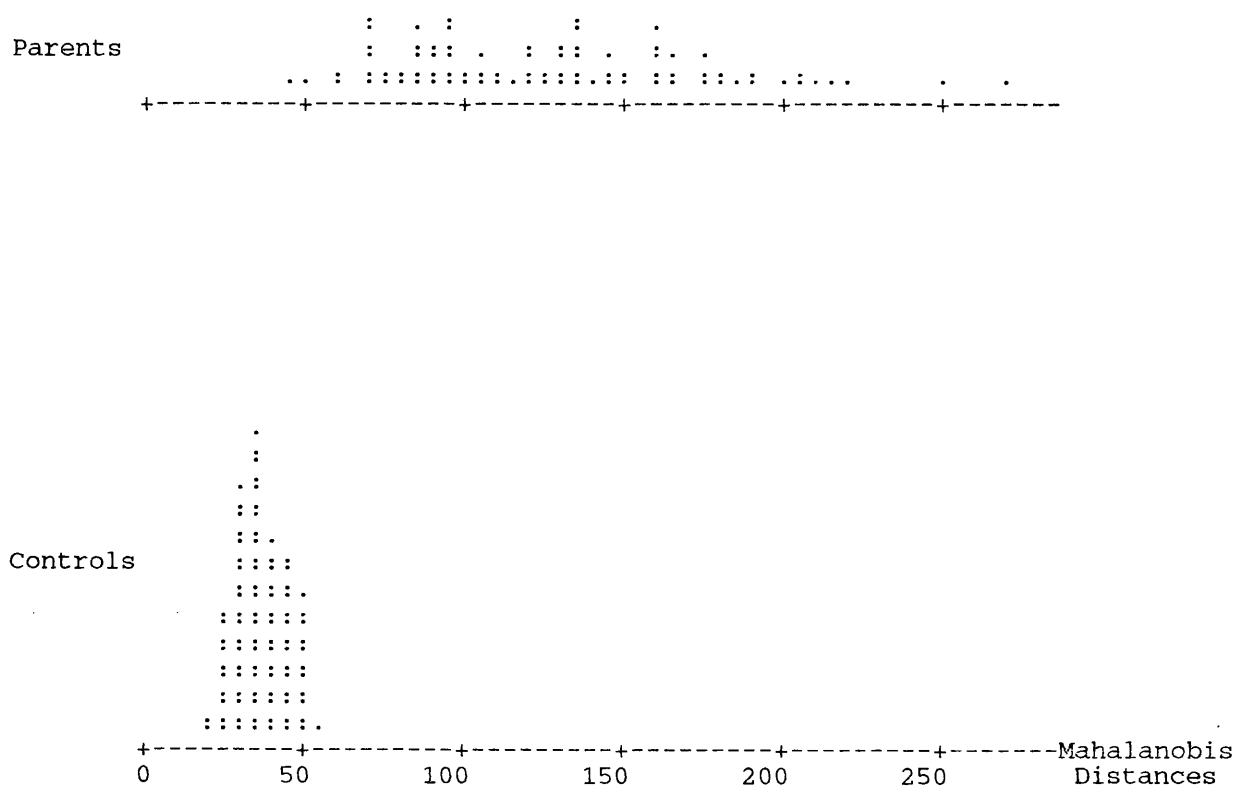


Figure 32 Dotplots Of Mahalanobis Distances for Parents vs Controls

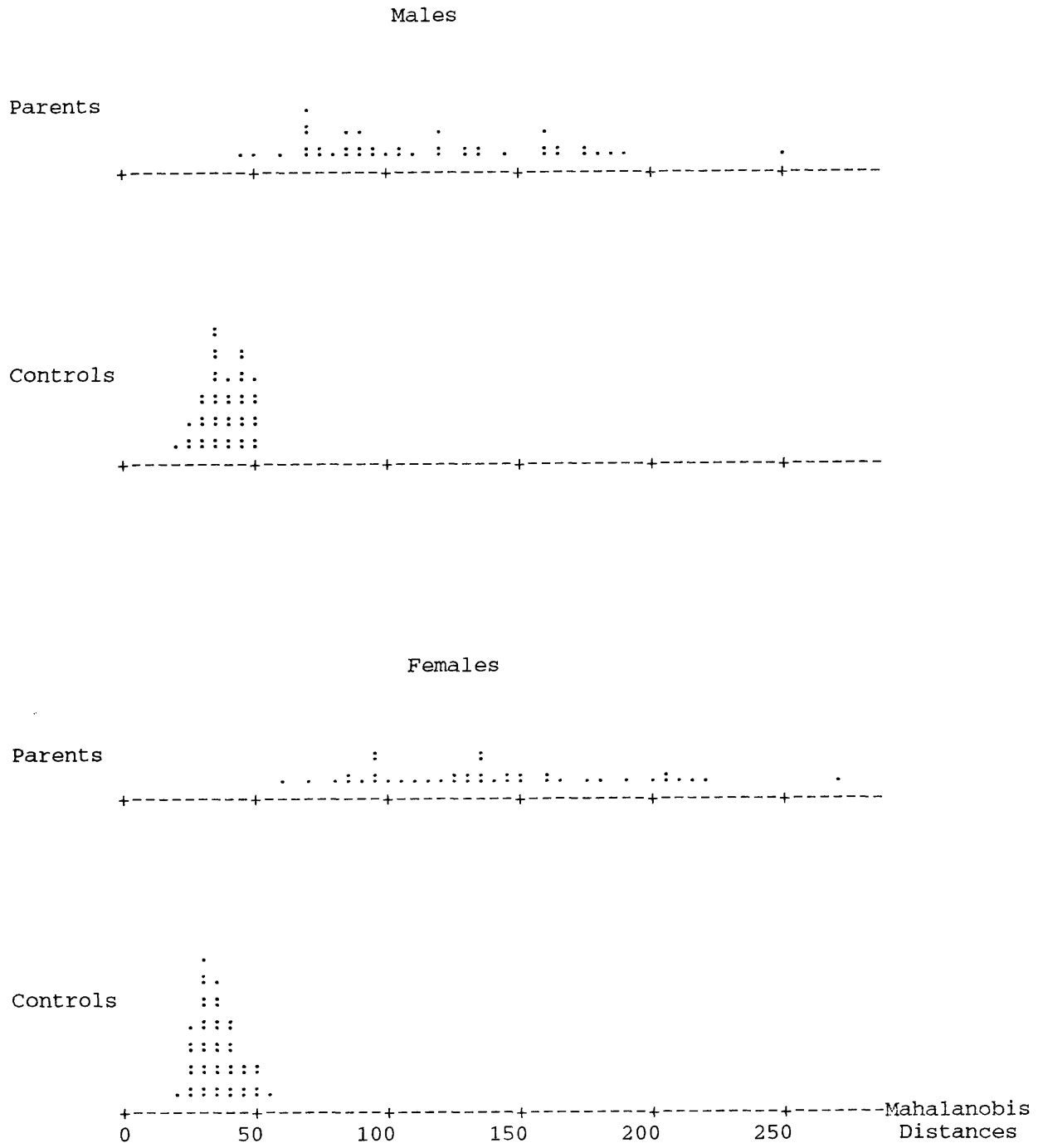


Figure 33 (a) Dotplots of Mahalanobis Distances Grouped by Sex

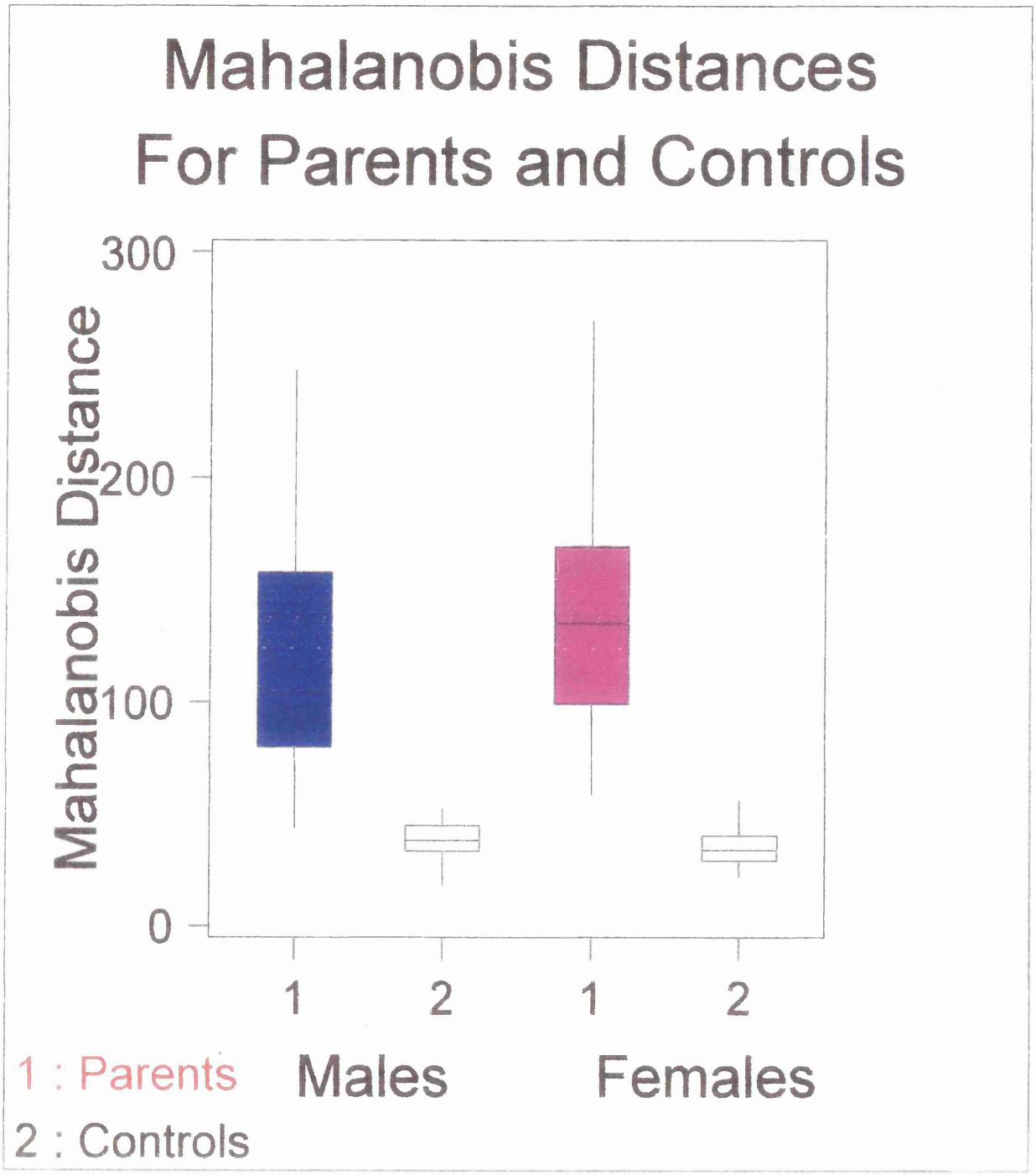


Figure 33 (b) Mahalanobis Distances for Parental and Control Groups Labelled by Sex

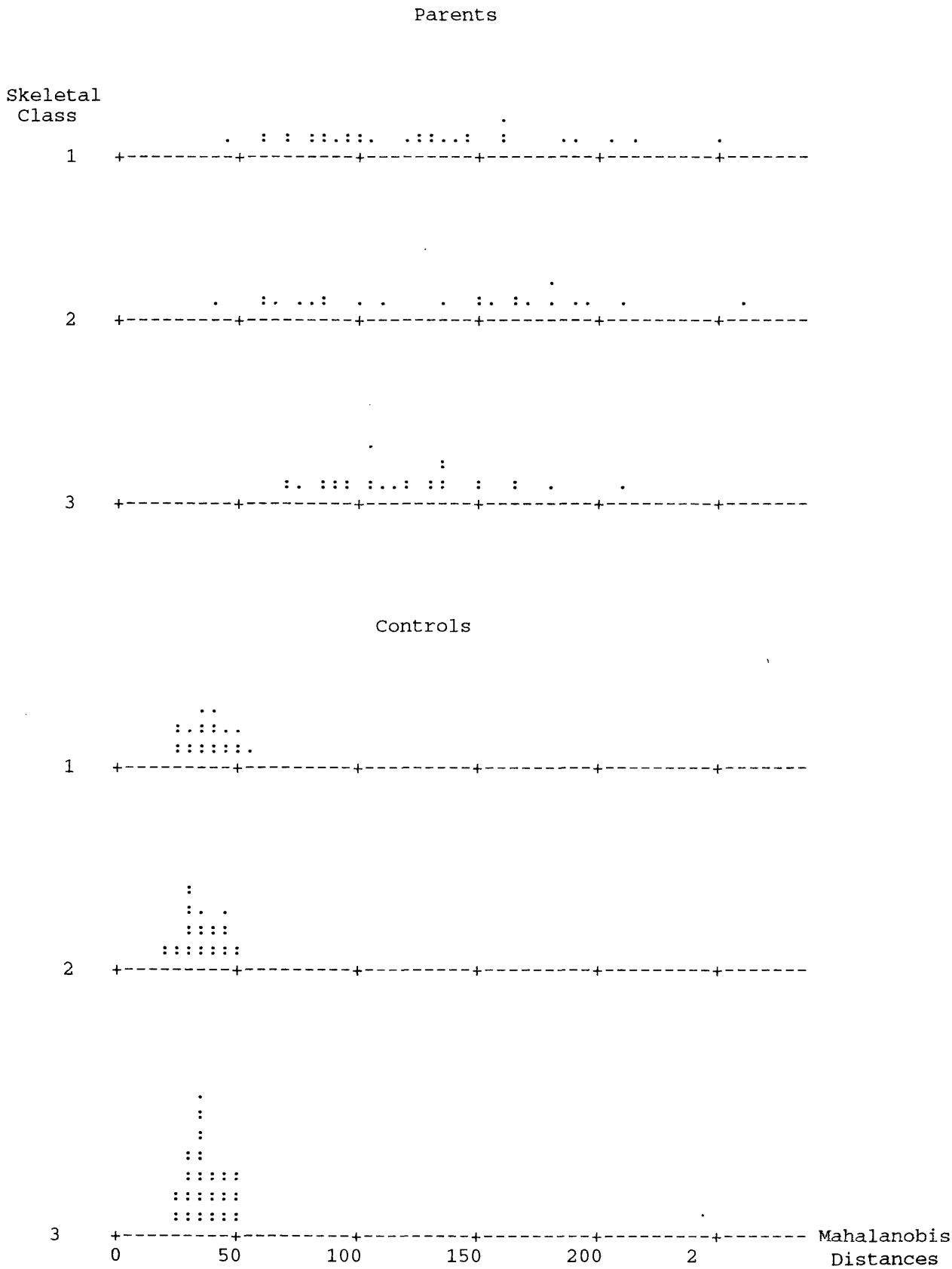


Figure 34 (a) Dotplots of Mahalanobis Distances for Parents and Controls Grouped by Skeletal Class

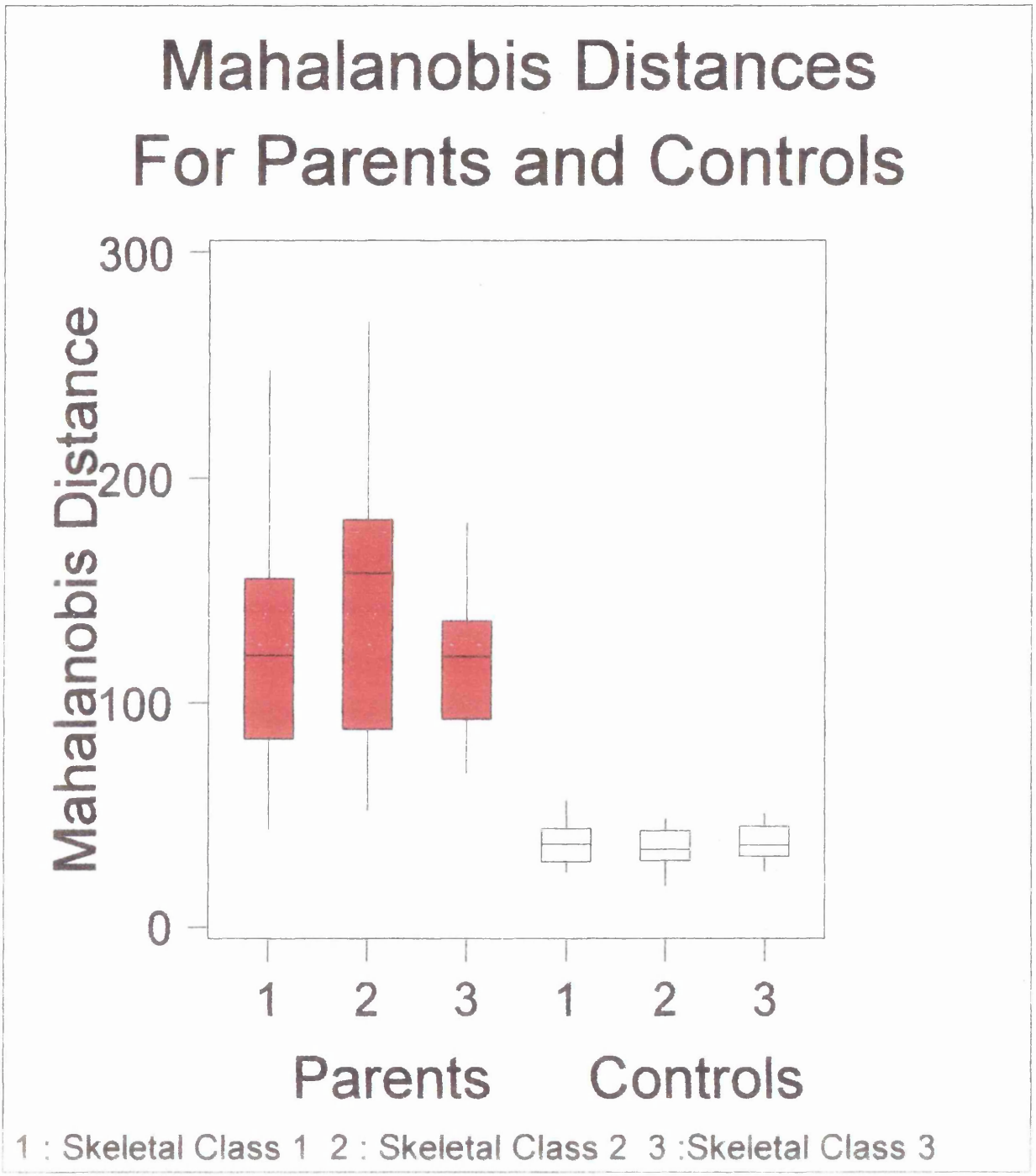


Figure 34 (b) Mahalanobis Distances for Parental and Contol Groups Labelled by Skeletal Clas

i) Males (Cephalometric mean plots: Figure 35 (a) and (b)).

(Histograms for significantly different parameters: Figure 36)

Compared to the control males, the fathers of children with CL(P) were shown to have reduced mandibular (Figure 36 (a)), symphyseal and maxillary areas (Figure 36 (b)) as measured on the lateral skull radiographs and a significant reduction in the palatal length (ANS to PNS) (Figure 36 (c)). In addition the cranial base angle (N-S-Ba) was significantly more acute (Figure 36 (d)) and the cross-sectional area of the cranium as measured on lateral skull radiographs was significantly smaller (Figure 36 (e)). The occipital subtenuce (see Figure 35 (b)) was however significantly larger in the fathers compared to the control males (Figure 36 (f)).

ii) Females (Cephalometric mean plots: Figure 37 (a) and (b)).

(Histograms for significantly different parameters: Figure 38)

Compared to the controls, the mothers of children with cleft lip and/or palate showed a significantly longer mandible measured from condylion to gnathion (Cd-Gn) and a significant increase in the anterior facial height (N-Me) and facial length as measured from sella to gnathion (S-Gn) (Figure 38 (a)). Both the anterior cranial base (S-N) and the clivus length (S-Ba) were significantly larger in the mothers (Figure 38 (b)). The cranial parameters showed a similar trend to the paternal group with a reduced cross-sectional area of the cranium (Figure 38(c)), and an increase in the occipital subtenuce length. However the frontal subtenuce was reduced in the maternal group (See Figure 37(b) and 38 (b)).

The 37 box and whisker plots illustrating the distribution for each variable are presented in Appendix C. These demonstrate the gender differences with the expected increase in size of male compared to female, the variation in craniofacial morphology according to cleft type for certain parameters as outlined in section 3.1.1, and the maternal/paternal differences highlighted above (section 3.1.3).

III) Stepwise discriminant analysis

Having identified these significant gender differences when comparing the cleft parents with a control, the final procedure in the analysis was to apply a discriminant analysis to the data. This was done in the expectation that certain

parameters in the male and female craniofacial morphology would be good discriminators of whether an individual of either gender possesses features which predispose them to having a child with cleft lip and/or cleft palate.

These results also proved to be highly significant. In the males those parameters found to be most useful as discriminators were cranial area (c6) and the parietal chord length (c2), cranial base angle (N-S-Ba), anterior face height (N-Me), ramus length (Cd-14) and the horizontal distance between the condyle position and the sella (s49) (which measures the antero-posterior position of the condyle relative to the cranial base). 90.5% of male parents were correctly classified (four cases were misclassified) as were 89.1% of controls (with five misclassified). Using a jackknifed classification rule 83.3% of parents and 82.6% of controls respectively were correctly classified.

For females the most useful discriminators were cranial area (c6), height of the cranium (V-Ba), the parietal chord length (c2) and both parietal and occipital subtenuce, (c8) and (c9) measurements respectively. The clivus length (S-Ba) and maxillary cross-sectional (s14) area were also useful discriminators. For this female group 95.1% of parents and 98% of controls were correctly classified with only two parents and one control case wrongly classified. Double checking using the jackknife analysis revealed similarly satisfactory discrimination with only three parents and one control misclassified. (92.7% of parents and 98% of controls correctly classified respectively.)

Summary

This study has shown by analysis of variance that the parameters which differentiated best between CP and CL(P) were mandibular length (Cd-Gn), ramus length(Cd-14), mandibular area (s2) and cranial area (c6), these being greater for CP. Furthermore the stepwise discriminant analysis showed that the one single parameter which discriminated best was the mandibular ramus length (Cd-14) being an accurate predictor in 71.4% of CP and 62.5% of CL(P) cases.

Table 18 Intervals and p-values from two sample t-tests for craniofacial parameters in male and female groups compared to their respective controls.

		Males		Females	
	Variable	Interval	p-value	Interval	p-value
s1	Ar-symp	0·22....0·59	0·0001**	-0·15....0·18	0·85
s2	Ar-mand	1·20....3·95	0·0003**	-0·54....1·93	0·27
s3	L-body	-2·63....2·63	0·64	-2·71....0·90	0·30
s7	Cd-Gn	-2·08....2·06	0·99	-4·44...-0·58	0·011*
s8	Cd-14	-0·14....4·22	0·067	-2·22....1·78	0·83
s11	Gonial ∠	-5·0.....0·37	0·09	-2·05....2·88	0·74
s14	Ar-max	0·176..0·72	0·0015**	-0·23....0·19	0·87
s15	ANS-PNS	0·68....3·40	0·0037**	-1·67....0·73	0·44
s18	Max-ht	-0·76....1·95	0·38	-1·09....1·01	0·94
s24	N-Me	-3·98....0·9	0·20	-4·87...-0·16	0·036*
s25	N-ANS	-2·41....0·25	0·11	-1·55....0·96	0·65
s26	Se-PNS	-1·73....0·92	0·54	-2·17....0·20	0·10
s27	S-Gn	-2·96....1·20	0·40	-4·92....0·91	0·005**
s30	PNS-Hor	-0·28....1·79	0·15	-0·55....1·48	0·36
s31	UFH:LFH	-1·38....0·59	0·42	-0·21....1·38	0·15
s32	A-N-S	-0·46....2·71	0·16	-1·38....1·37	0·99
s33	A-N-B	-0·68....2·34	0·28	-1·24....1·41	0·90
s35	MMPA	-3·64....1·7	0·47	-2·63....1·85	0·73
s39	SN-Or	-1·96....1·85	0·95	-1·70....1·54	0·92
s40	SN-FP	-1·50....1·14	0·79	-2·07....0·81	0·38
s41	Ar-CBase	-0·29....0·67	0·44	-0·46....0·44	0·96
s42	S-N	-1·47....1·16	0·81	-2·56....0·27	0·016*
s44	S-Ba	-1·31....0·92	0·73	-2·35....0·10	0·033*
s47	44-47	-0·69....0·36	0·53	-0·92....0·00	0·048*
s49	Cd-56	-1·53....0·78	0·52	-0·67....1·20	0·58
s50	S-56	-2·08....0·37	0·17	-1·27....0·68	0·55
s51	FMN-N	-0·10....0·11	0·93	-0·06....0·11	0·54

Table 18 (continued) Intervals and p-values from two sample t-tests for craniofacial parameters in male and female groups compared to their respective controls.

		Males		Females	
	Variable	Interval	p-value	Interval	p-value
c1	N-Br	-0·34....3·83	0·10	-2·16....1·97	0·93
c2	Br-L	-2·10....1·71	0·84	-3·23....1·30	0·38
c3	L-Op	-4·0.....1·47	0·36	-5·49...-0·56	0·02
c4	V-Ba	-2·55....1·43	0·58	-4·17....0·18	0·07
c5	Gla-CPo	-1·89....3·32	0·59	-3·97....0·73	0·17
c6	Ar-Cranium	8·5....20·7	0·00***	6·1....17·10	0·0001***
c7	from C5	-0·07....2·16	0·07	0·55....2·74	0·004**
c8	from C11	-1·23....0·90	0·76	-0·43....2·02	0·20
c9/c10	from C19	-4·25....0·08	0·04	-5·33...-1·90	0·0001***

Note: Ar denotes area measurements.

c7,c8 and c9/c10 are frontal, parietal and occipital subtenuce measurements respectively.

Interval figures refer to control minus parental values for each parameter, therefore;
- = parents larger
+ = controls larger

* $p < 0·05$

** $p < 0·01$

*** $p < 0·001$

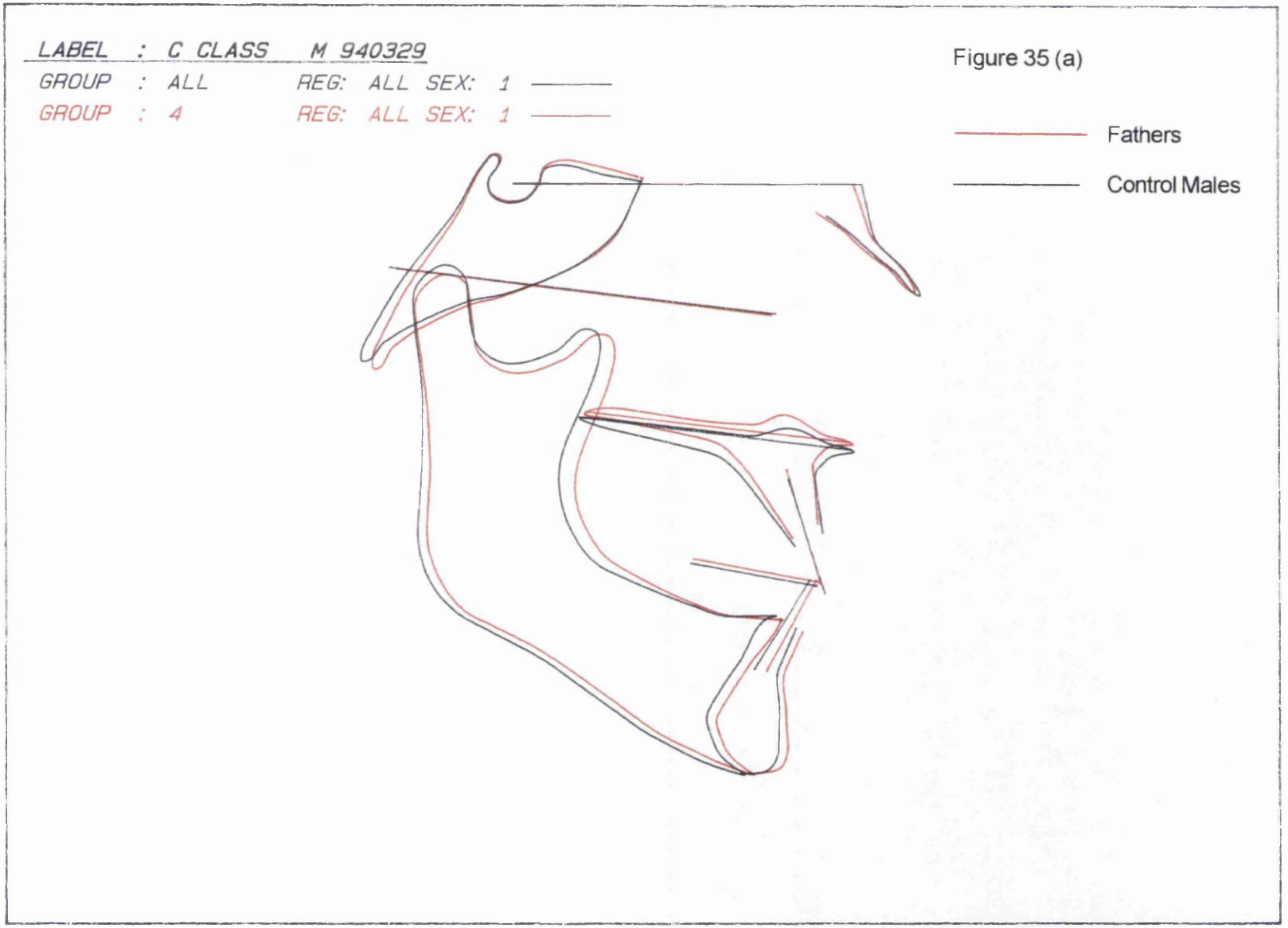


Figure 35 (a) Superimposition of mean facial plots for fathers versus control males

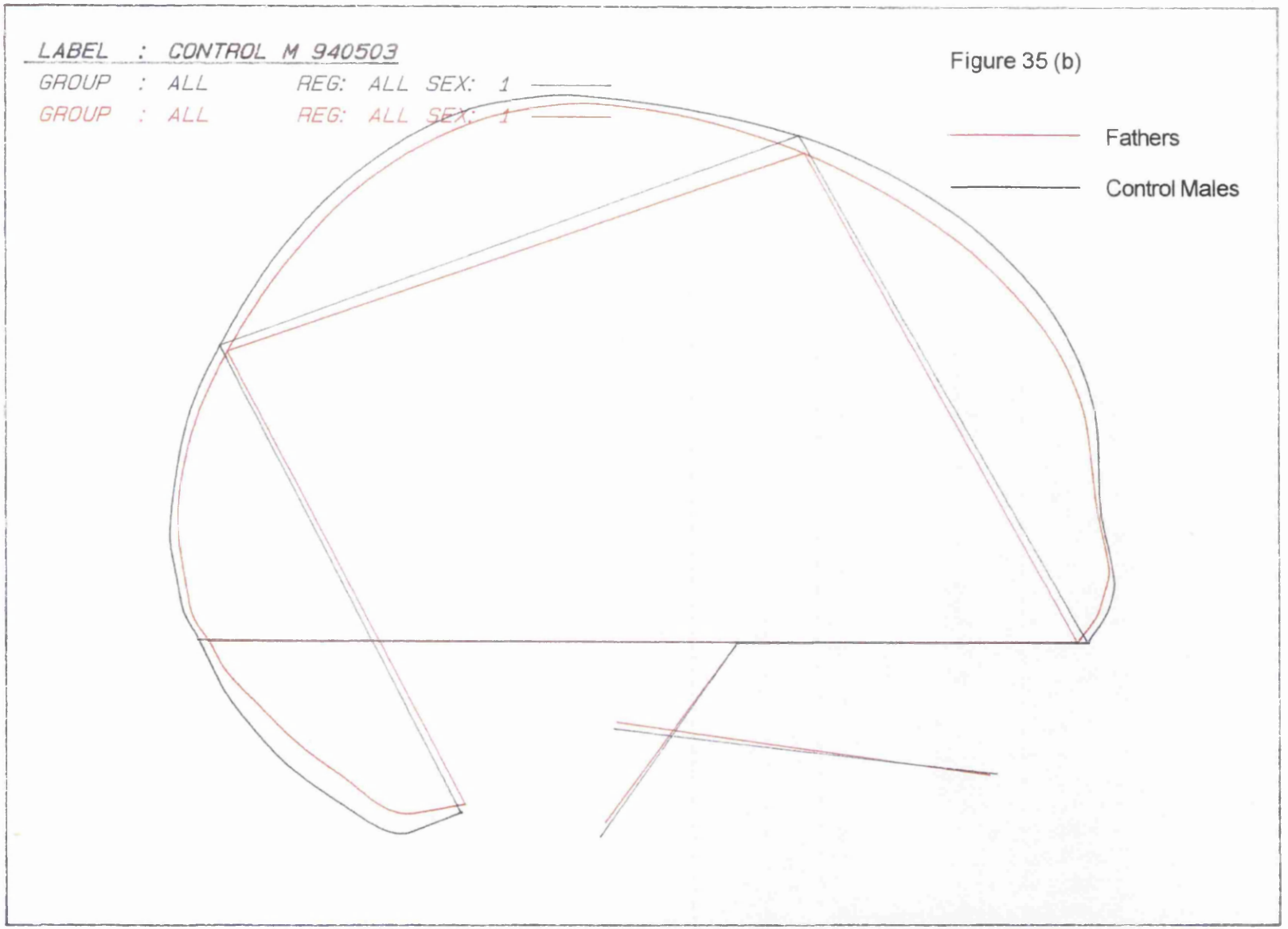
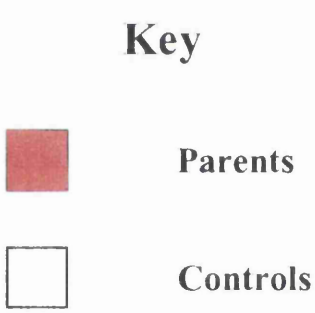


Figure 35 (b) Cranial analysis: superimposition of mean cranial plots for fathers versus control males

Figure 36: (a) - (f)

Histograms of Significant Craniofacial Differences Between Fathers and Male Controls



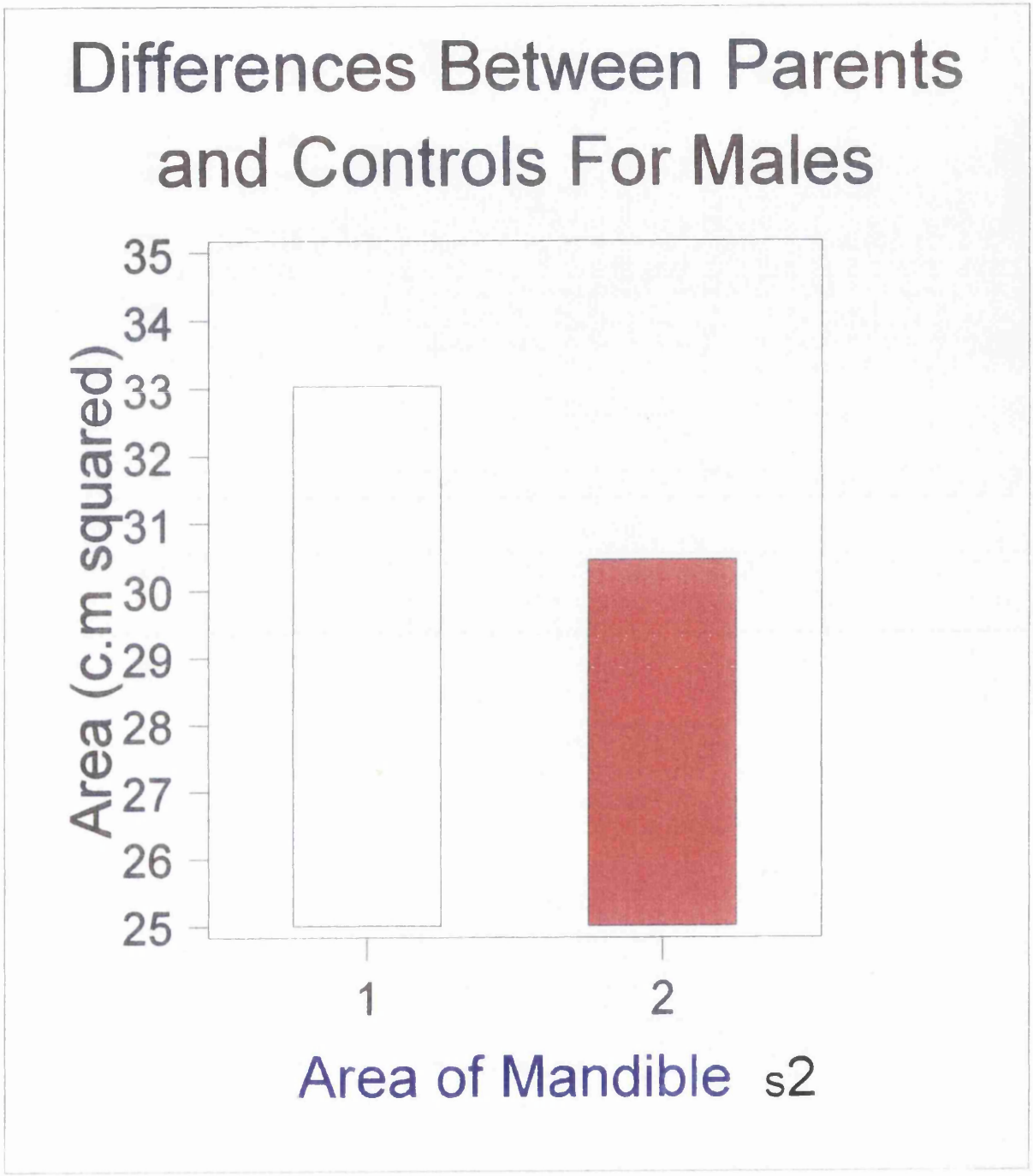

Differences Between Parents and Controls For Males
Area (c.m squared)
35
34
33
32
31
30
29
28
27
26
25
1
2
Area of Mandible s2

Figure 36 (a)

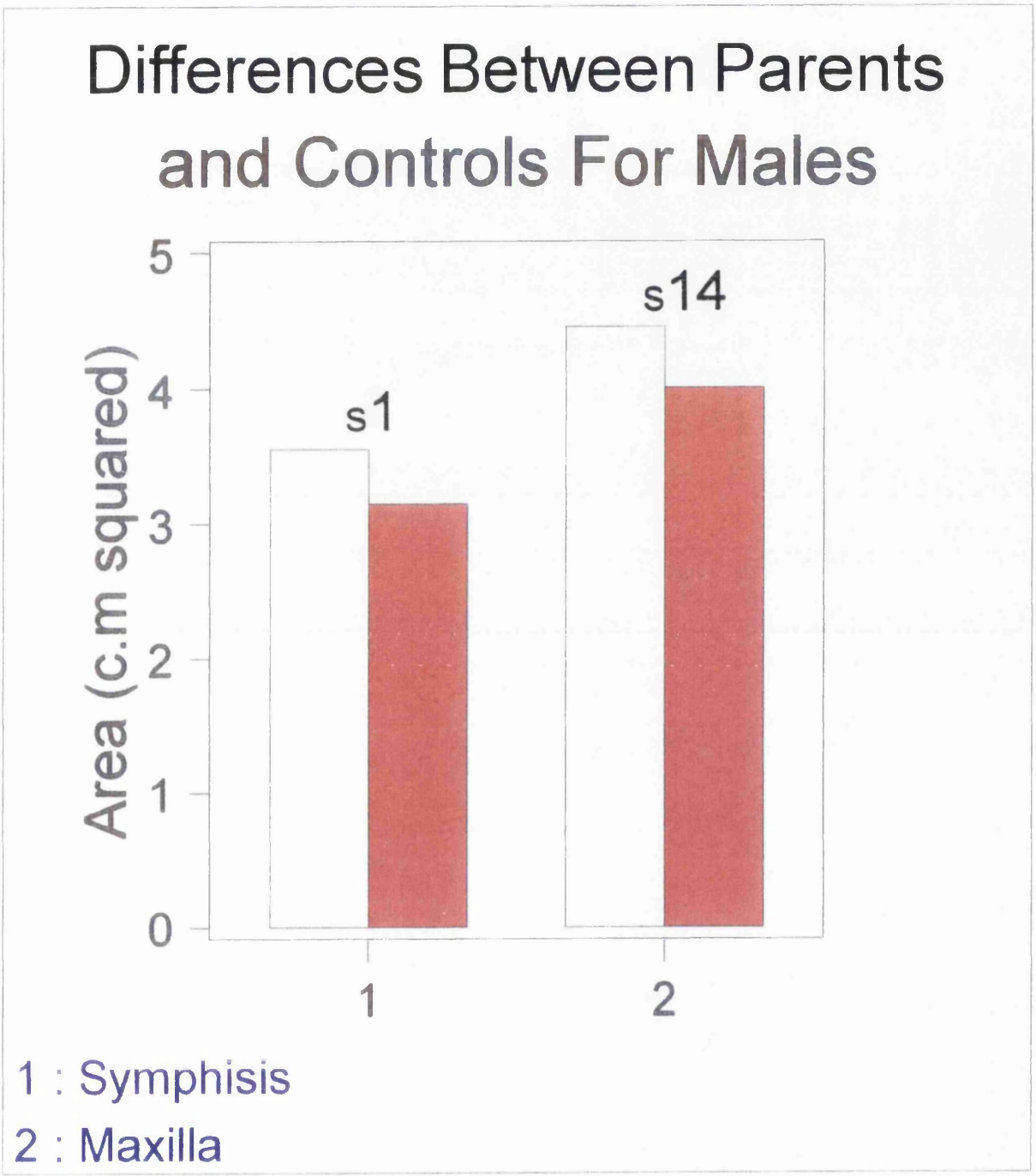

Differences Between Parents and Controls For Males
Area (c.m squared)
0
1
2
3
4
5
s1
s14
1
2
1 : Symphisis
2 : Maxilla

Figure 36 (b)

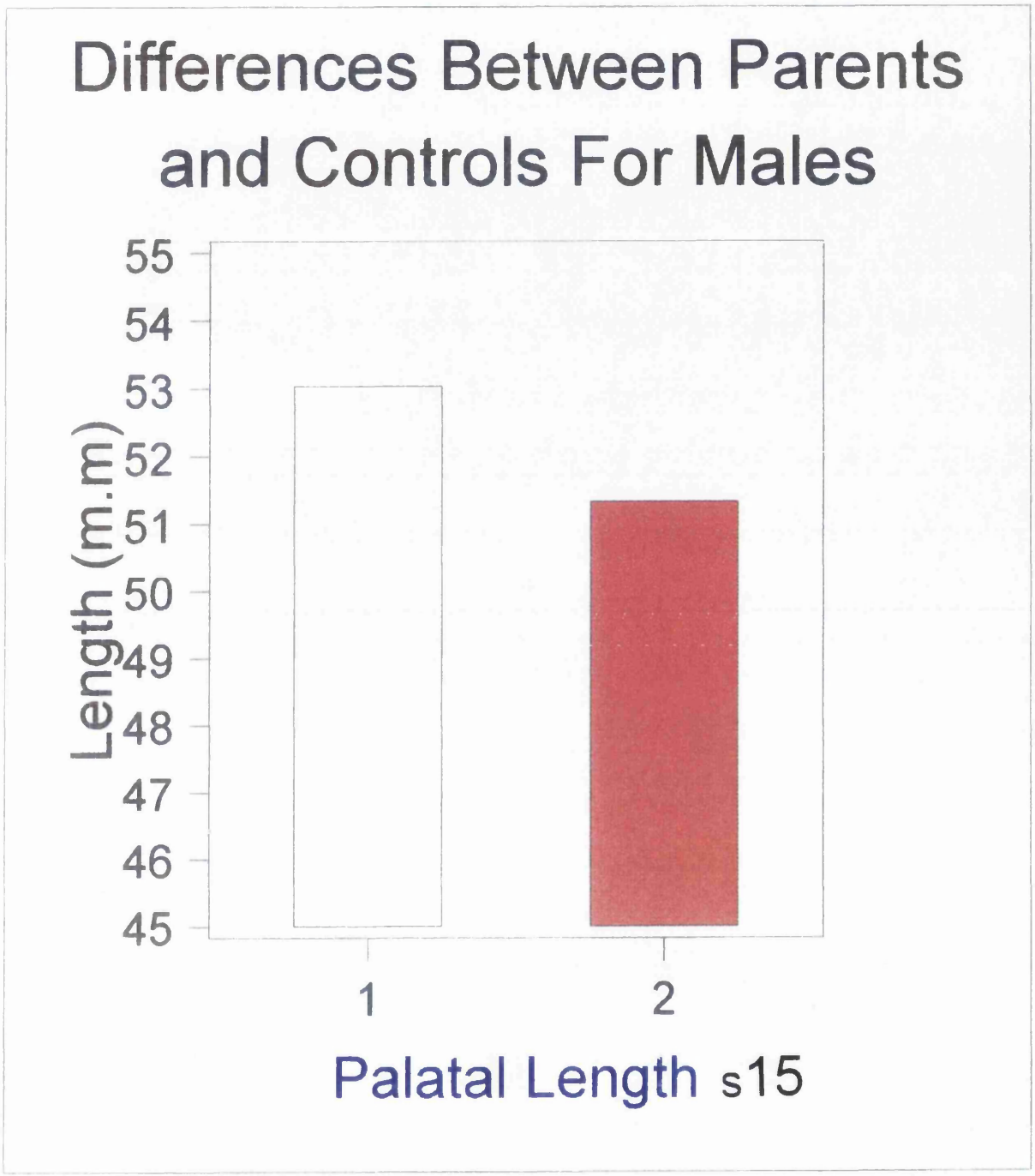

Differences Between Parents and Controls For Males
Length (m.m)
55
54
53
52
51
50
49
48
47
46
45
1
2
Palatal Length s15

Figure 36 (c)

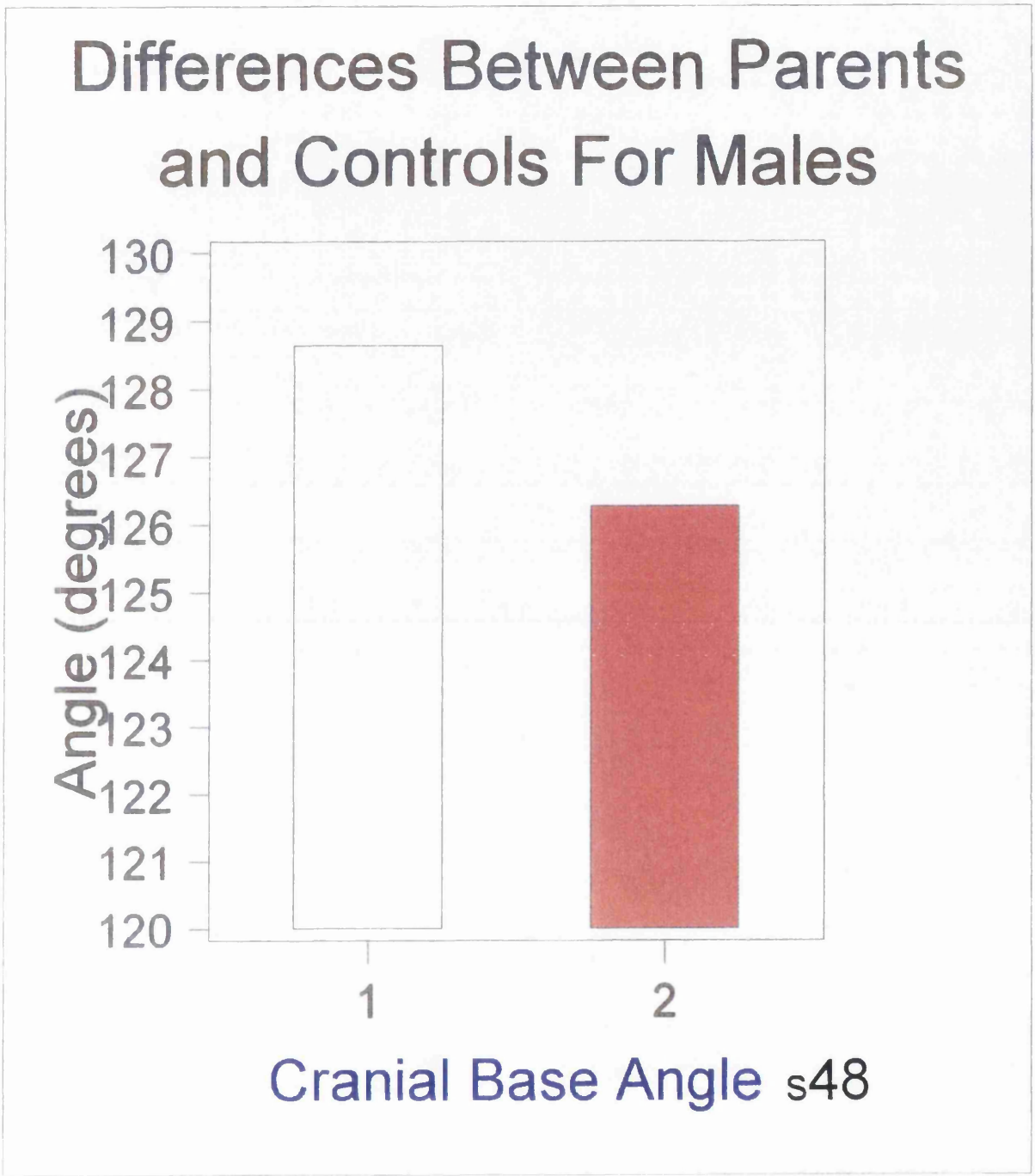

Differences Between Parents and Controls For Males
Angle (degrees)
130
129
128
127
126
125
124
123
122
121
120
1
2
Cranial Base Angle s48

Figure 36 (d)

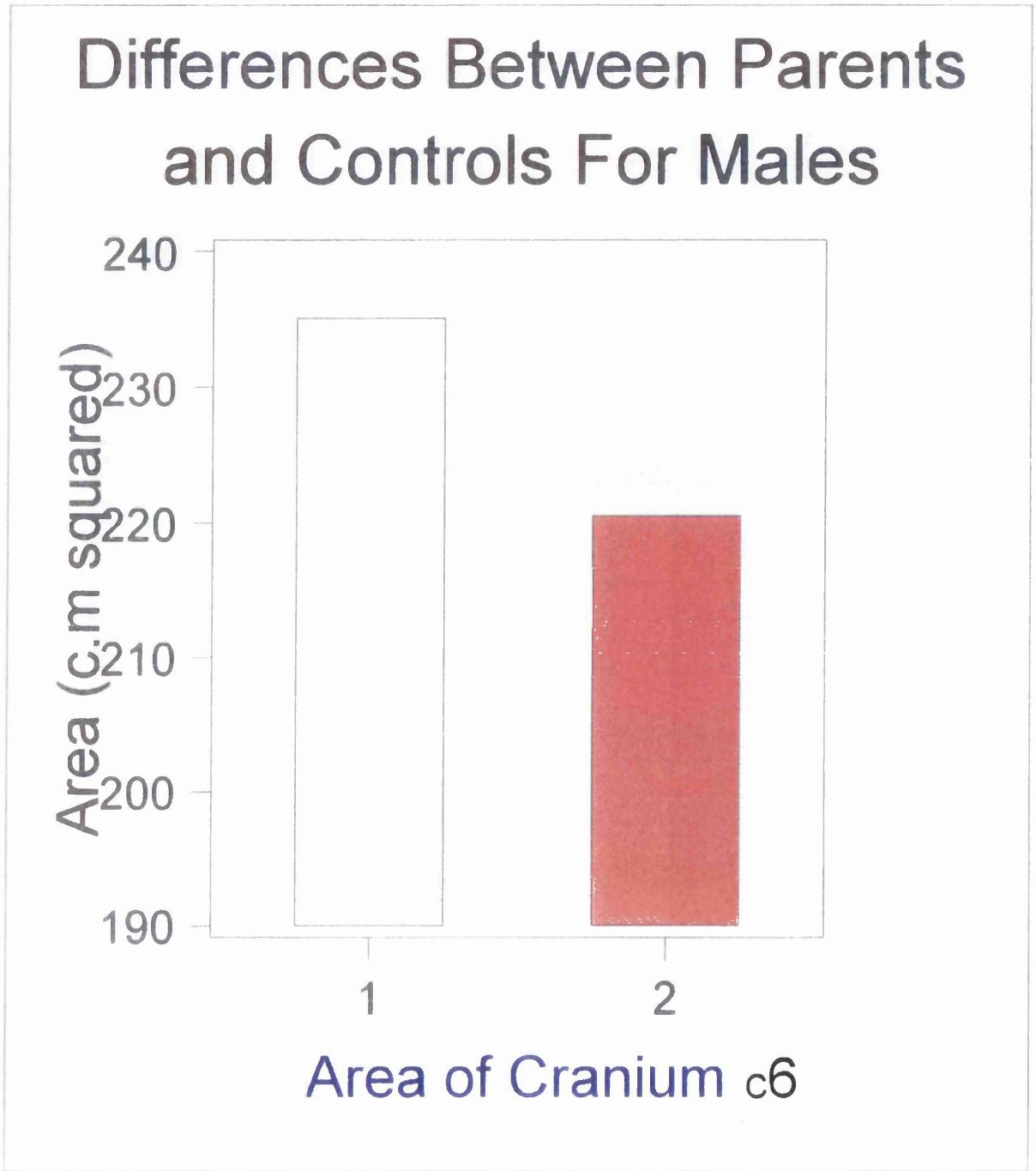

Differences Between Parents and Controls For Males
240
230
220
210
200
190
Area (c.m squared)
1
2
Area of Cranium c6

Figure 36 (e)

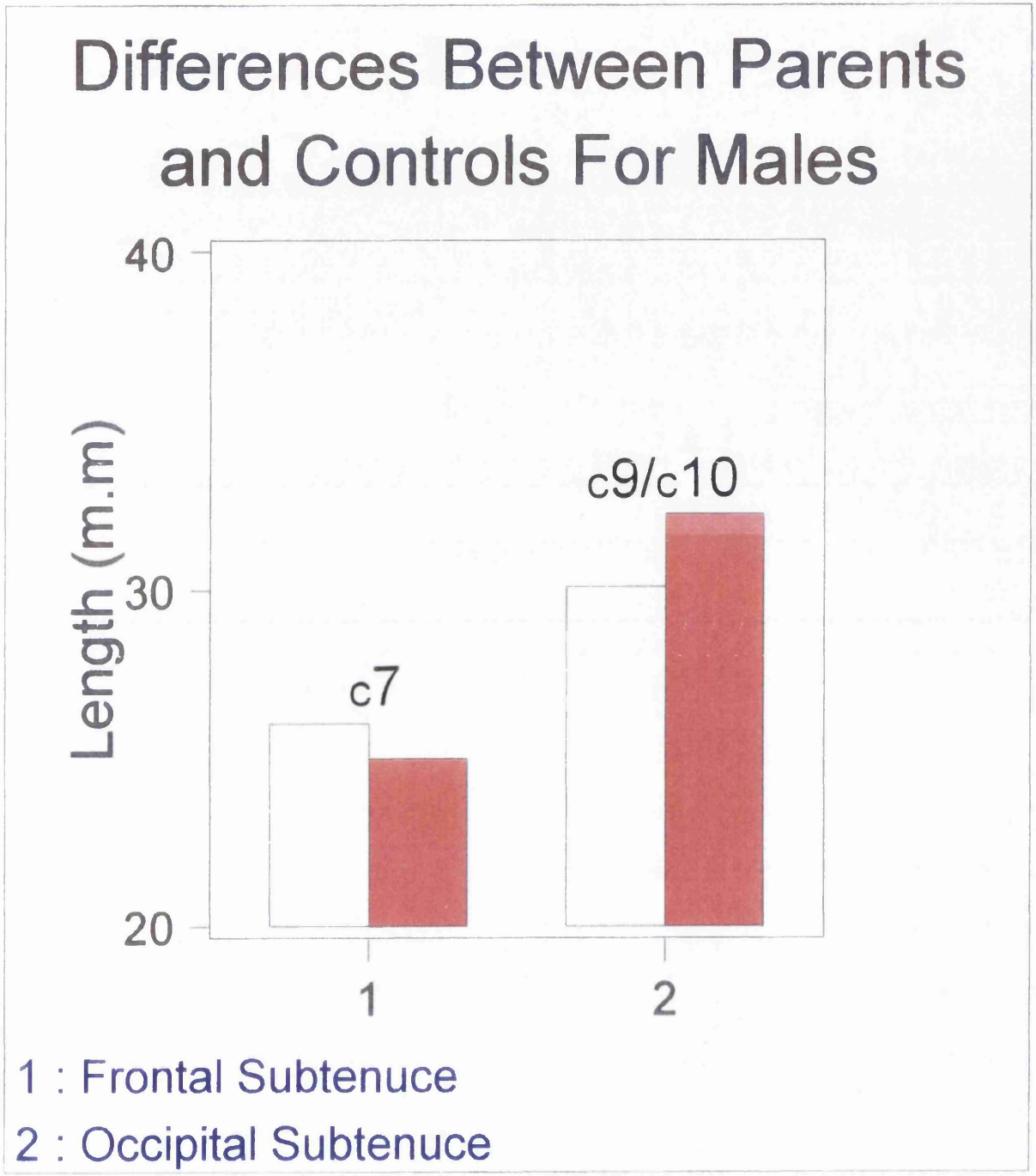

Differences Between Parents and Controls For Males
40
30
20
Length (m.m)
c7
c9/c10
1
2
1 : Frontal Subtenuce
2 : Occipital Subtenuce

Figure 36 (f)

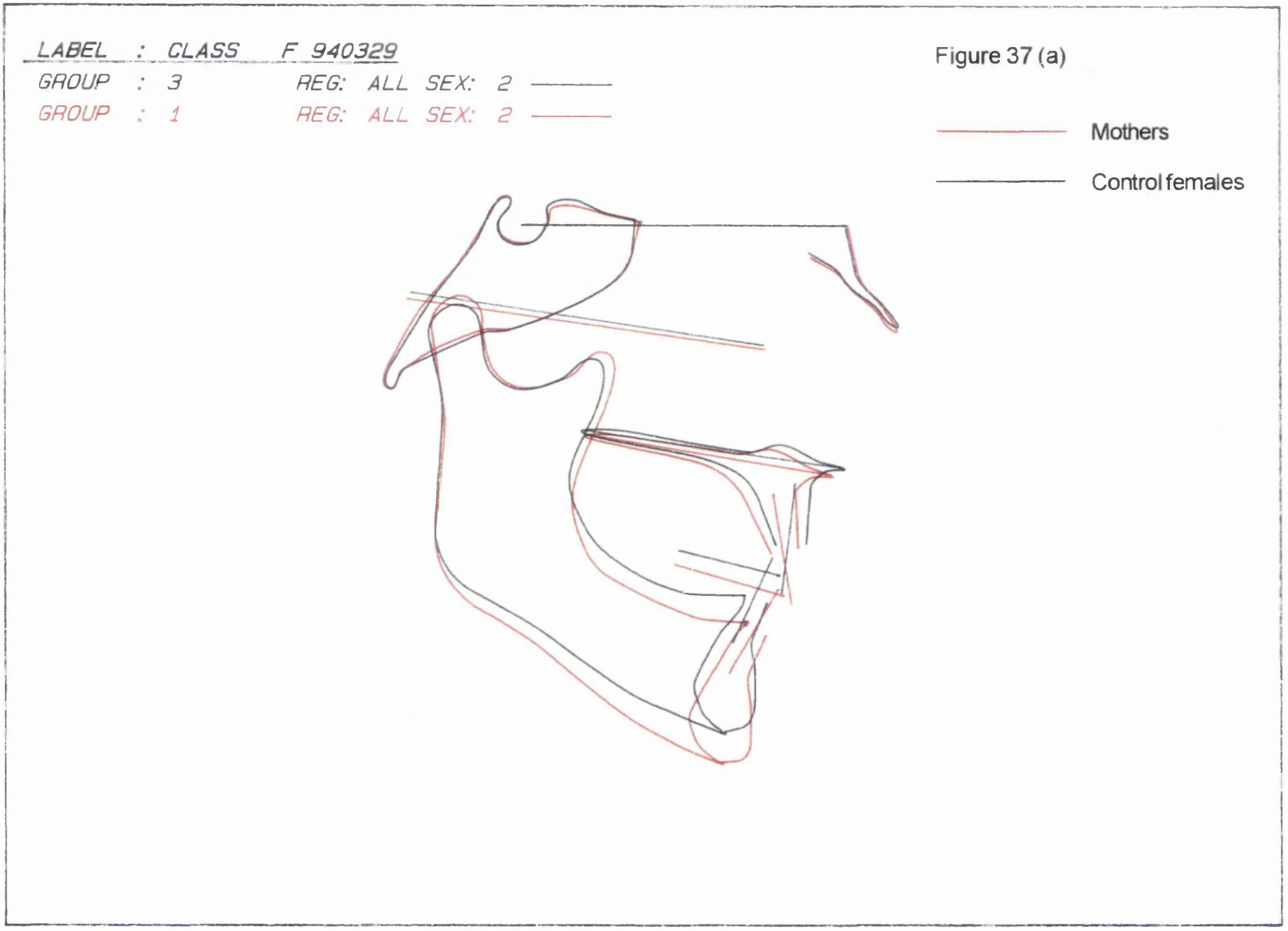


Figure 37 (a) Superimposition of mean facial plots for mothers versus control females

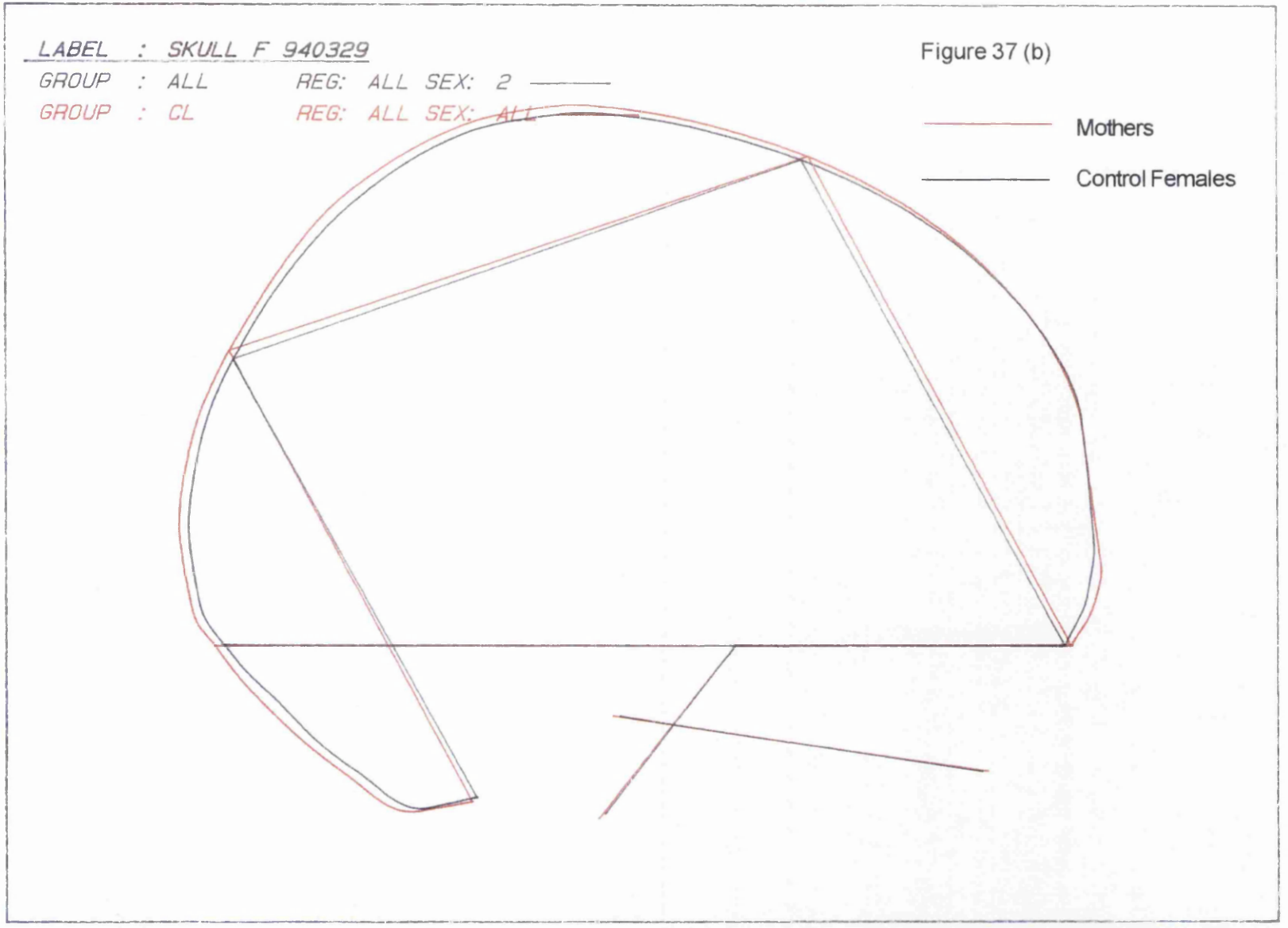
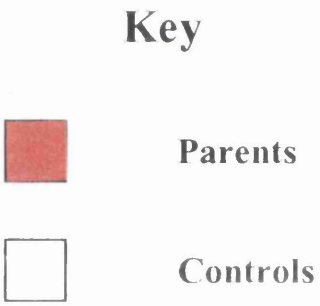


Figure 37 (b) Superimposition of mean cranial plots for mothers versus control females

Figure 38: (a) - (c)

Histograms of Significant Craniofacial Differences Between Mothers and Female Controls



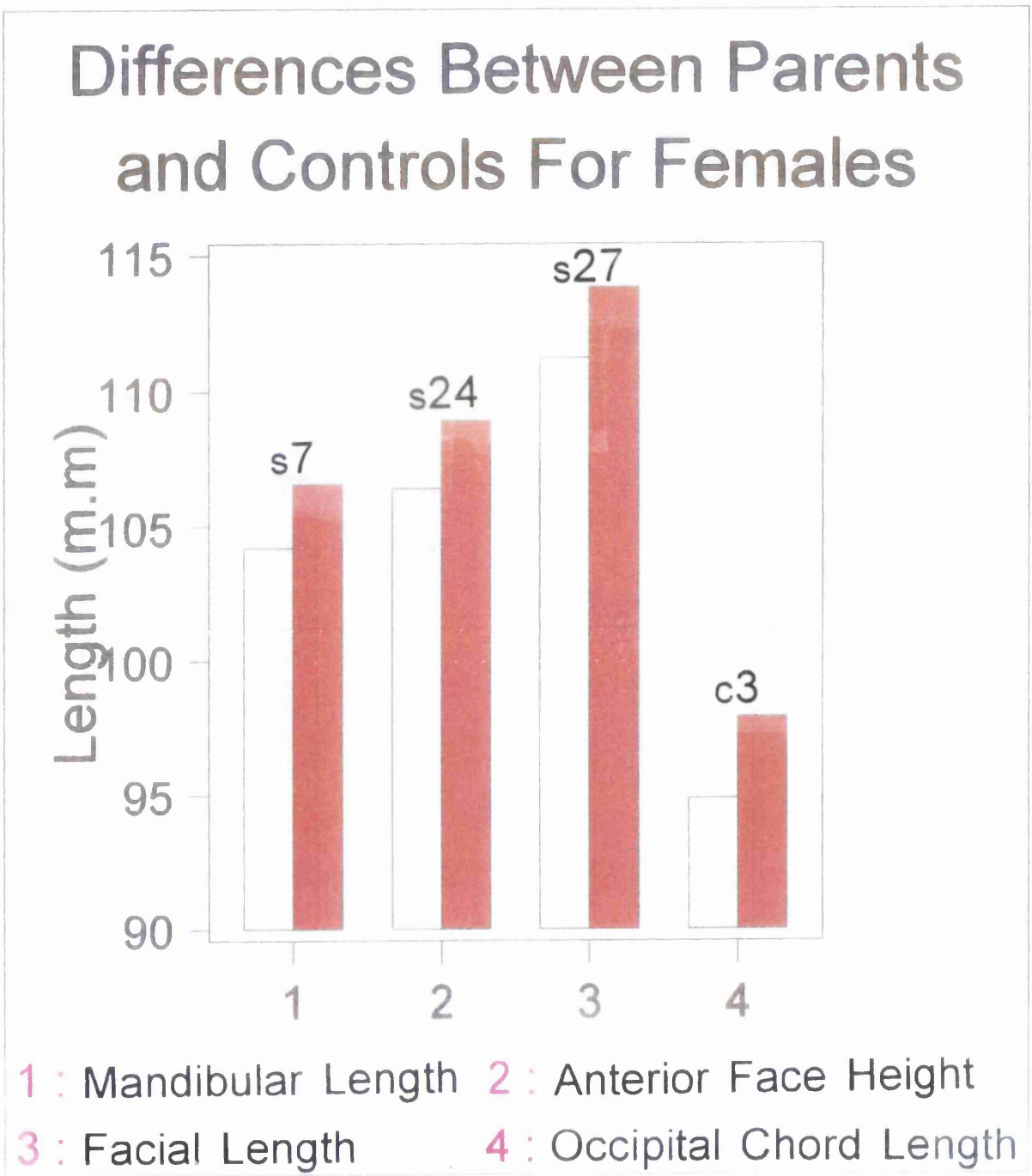

Differences Between Parents and Controls For Females
Length (m.m)
115
110
105
100
95
90
s7
s24
s27
c3
1
2
3
4
1 : Mandibular Length
2 : Anterior Face Height
3 : Facial Length
4 : Occipital Chord Length

Figure 38 (a)

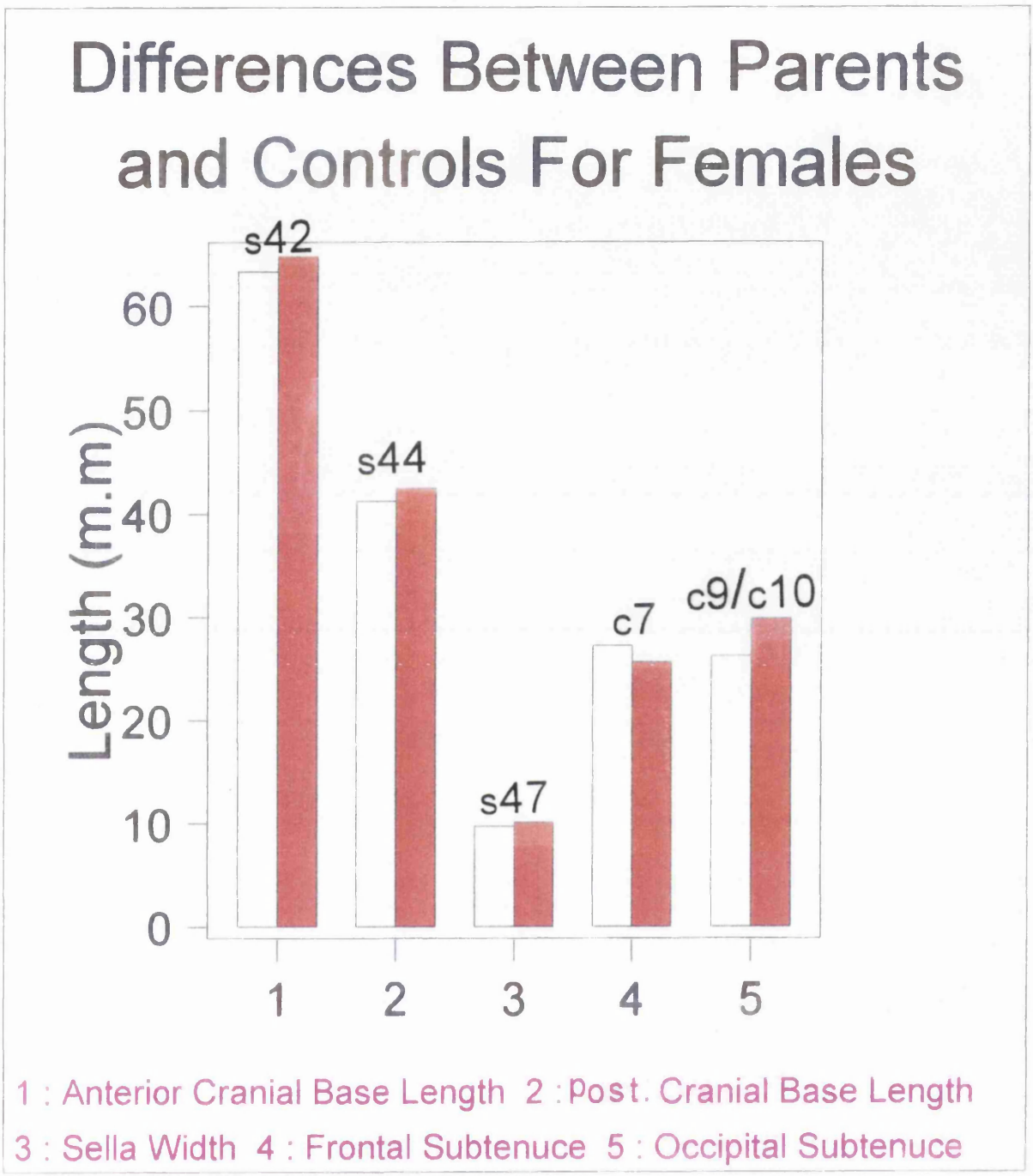

Differences Between Parents and Controls For Females
Length (m.m)
0
10
20
30
40
50
60
s42
s44
s47
c7
c9/c10
1
2
3
4
5
1 : Anterior Cranial Base Length 2 : Post. Cranial Base Length
3 : Sella Width 4 : Frontal Subtenuce 5 : Occipital Subtenuce

Figure 38 (b)

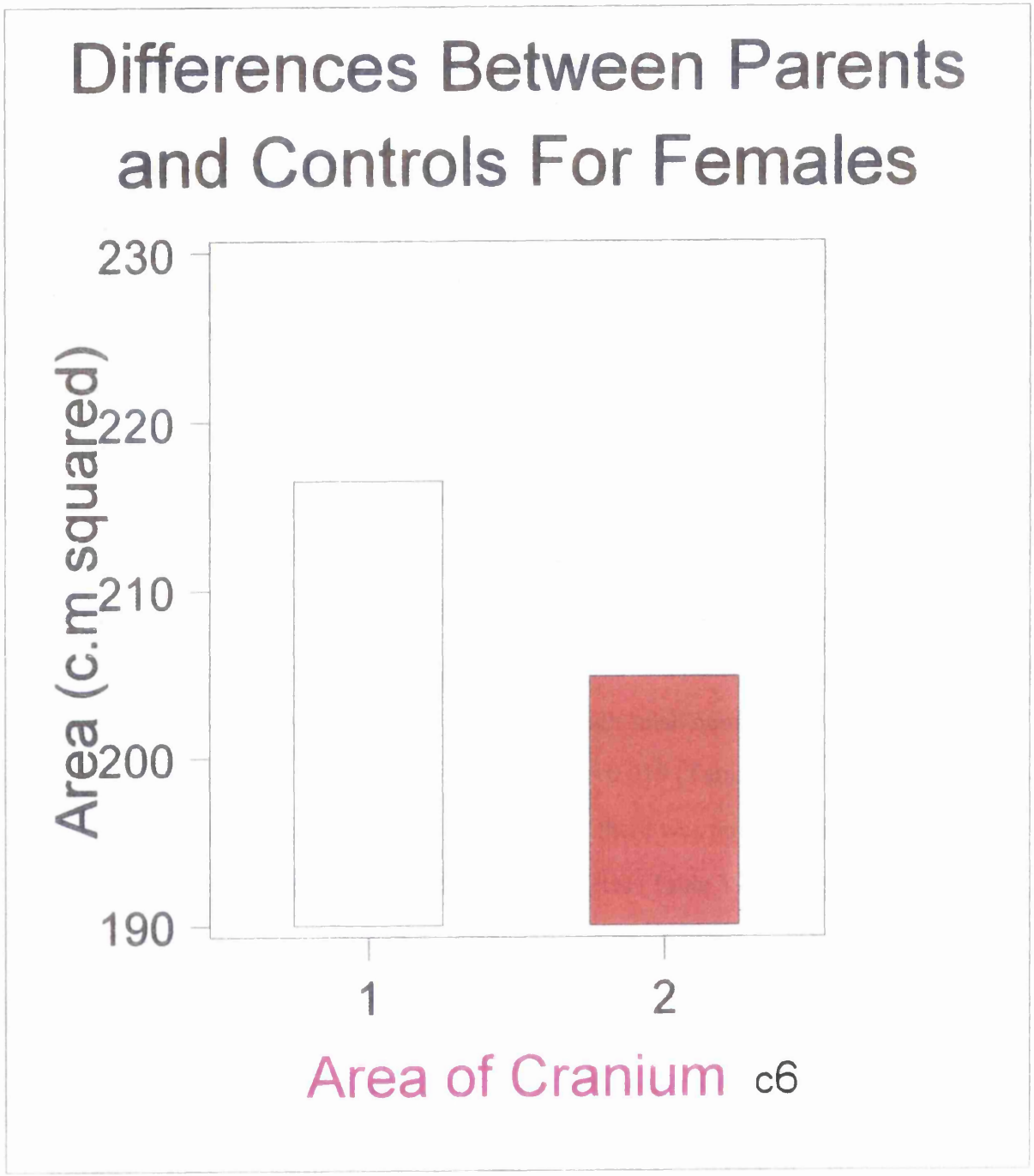

Differences Between Parents and Controls For Females
230
220
210
200
190
Area (c.m squared)
1
2
Area of Cranium c6

Figure 38 (c)

3.2 GENETIC RESULTS

3.2.1 Comparison of allele and genotype frequency

(a) Allele frequency

The results of the statistical comparison of the parental and control allele frequency data using the Chi-squared test (χ^2) are given in table 19. Table 19(a) shows that there was a tendency towards an increased frequency of the TGFα/*Bam*HI A1 (10 kb) allele in the CP parents which just failed to reach statistical significance ($\chi^2 = 3.82$ p = 0.051). This feature was not apparent for the CL(P) parental group ($\chi^2 = 1.24$ p = 0.265) nor for either group of probands. There was a highly significant difference between the two cleft groups ($\chi^2 = 7.154$ p = 0.0075) in respect of this A1 allele frequency.

There was a significantly increased frequency of the TGFα/*Taq*I 1.7 kb allele (C2) in CL(P) parents relative to the control frequency ($\chi^2 = 5.06$ p = 0.024). The frequency of the C2 allele is also significantly increased in the CP parental group ($\chi^2 = 6.17$ p = 0.013) but comparison of CL(P) with CP parents revealed no significant difference ($\chi^2 = 0.085$ p = 0.77) Table 19(c). C2 allele frequency in affected probands in both cleft categories also shows a statistically significant increase compared to the control but it is not possible to draw meaningful conclusions from such small numbers (for CL(P) n = 11: $\chi^2 = 4.48$, p = 0.034 and for CP n = 8: $\chi^2 = 5.42$, p = 0.019 (Table 20).

When comparing parental and control groups, there was no significant difference in frequency of either of the *Rsa*I derived B1 or B2 alleles (Table 19(b)), nor was there any difference in the A2 or C1 allele frequencies compared to the mean control figures. It is also apparent from the above figures that the A1 and the C2 allele which has an increased prevalence in the cleft parent population compared to the control are the least common in the general population. Control population frequency figures for the *Bam*HI A1 allele is 13.3% and for the *Taq* C2 allele 4.16% (Holder *et al.*, 1992).

Table 19 Allele frequency in parental sample.

(a) Polymorphisms identified by restriction with *Bam*HI

	A1	A2	Relative Risk (R)		χ^2	p-value
			A1	95% CI		
Holder *et al.*#	16	104				
CP	15	45	2·17	1·00..... 4·71	3·82	0·050
CL(P)	6	68	0·57	0·22..... 1·52	1·24	0·265
All Clefts	21	113	1·21	0·60..... 2·44	0·28	
CP v CL(P)					7·154	0·0075**

(b) Polymorphisms identified by restriction with *Rsa*I

	B1	B2	Relative Risk (R)		χ^2	p-value
			B2	95% CI		
Holder *et al.*	42	76				
CP	18	40	1·17	0·60..... 2·3	0·36	0·549
CL(P)	25	49	1·08	0·59..... 2·00	0·07	0·798
All Clefts	43	89	1·14	0·68..... 1·93	0·253	0·615
CL v CL(P)					0·112	0·738

(c) Polymorphisms identified by restriction with *Taq*I

	C1	C2	Relative Risk (R)		χ^2	p-value
			C2	95% CI		
Holder *et al.*	115	5				
CP	53	9	3·91	1·33.... 11·45	6·167	0·013*
CL(P)	68	10	3·38	1·17..... 9·78	5·056	0·024*
All Clefts	121	19	3·61	1·38..... 9·47	6·82	0·009**
CL v CL(P)					0·085	0·771

* $p < 0·05$

** $p < 0·01$

control data reproduced by kind permission of the following authors:

Holder, S.E., Vintiner, G.M., Farren, B., Malcolm, S., .Winter, R.M., (1992)

Table 20 Allele frequency in proband sample

(a) Polymorphisms identified by restriction with *Bam*HI

	A1	A2	χ^2	p-value
Holder *et al.*	16	104		
CP	2	14	0·01	0·926
CL(P)	1	19	1·12	0·290
All Clefts	3	33	0·65	0·420
CP v CL(P)			0·655	0·418

(b) Polymorphisms identified by restriction with *Rsa*I

	B1	B2	χ^2	p-value
Holder *et al.*	42	76		
CP	4	14	1·24	0·264
CL(P)	7	15	0·12	0·730
All Clefts	11	29	0·88	0·350
CP v CL(P)			0·457	0·499

(c) Polymorphisms identified by restriction with *Taq*I

	C1	C2	Relative Risk (R)		χ^2	p-value
			C2	95% CI		
Holder *et al.*	115	5				
CP	13	3	5·31	1·30 .... 21·63	5·42	0·019*
CL(P)	15	3	4·60	1·12 .... 18·91	4·48	0·034*
All Clefts	28	6	4·93	1·54 .... 15·73	7·26	0·007**
CP v CL(P)					0·025	0·874

* $p < 0·05$

** $p < 0·01$

(b) Genotype frequency

Having established the association between the parental *Taq*I C2 allele frequency with clefting and of the increased *Bam*HI A1 allele frequency with CP compared to CL(P) further genetic investigation was carried out. Parental genotypes for each of the three restriction enzymes were recorded and analysed to establish the relationship between parental genotype and their child's birth defect. For the purpose of the initial analysis both fathers and mothers were grouped together in a "parental" group, and although there were very small numbers (n = 19), the proband genotype frequency was also examined.

1(a) *Bam*HI genotypes v cleft type. (Table 21(a))

On digestion with *Bam*HI the A2 allele is predominant as expected since the A1 allele presents in only a small percentage of the normal (control) population. Homozygosity for the A1 allele was rare in all previous studies and was unrepresented in any of the parent or control subjects in the present study. The chi-squared test statistic was therefore applied to comparison of the A1A2 and A2A2 genotypes only with one degree of freedom.

Analysis of the cleft group overall versus the control showed no difference in genotype frequency ($\chi^2 = 0.04$, $p = 0.56$), but subdivision of the cleft group into CP and CL(P) did reveal significant differences. The CP parents were found to have a significantly higher frequency of the A1A2 genotype compared to the control ($\chi^2 = 4.82$, $p = 0.028$), and the difference between the CP and CL(P) groups for the prevalence of the A1A2 genotype was highly statistically significant ($\chi^2 = 7.29$, $p = 0.003$). The A1A2 genotype was somewhat under represented in both the CL(P) parental and proband groups compared to the control, but not to the level of statistical significance; while the increased frequency of the A1A2 genotype recorded for the CP parents was not reflected in the small sample of CP probands involved in the present study. (Table 22(a))

Table 21 Parental genotype frequency.

(a) *Bam*HI genotypes.

	A1A1	A1A2	A2A2	Relative Risk (R)		χ^2	p-value
				A1A2	95% CI		
Holder *et al.*	0	16	44				
CP	0	15	15	2·75	1·11..... 6·78	4·82	0·028*
CL(P)	0	6	31	0·53	0·19..... 1·50	1·42	0·23
All clefts	0	21	46	1·26	0·58..... 2·71	0·04	0·56
For A1A2 CP v CL(P)						7.29	0.003*

(b) *Rsa*I genotypes.

	B1B1	B1B2	B2B2	χ^2	p-value
Holder *et al.*	10	22	27		
CP	2	13	14	1·74	0·42
CL(P)	4	15	19	0·78	0·68
All clefts	6	28	33	1·82	0·40

(c) *Taq*I genotypes.

	C1C1	C1C2	C2C2	Relative Risk (R)		χ^2	p-value
				C2#	95% CI		
Holder *et al.*	55	5	0				
CP	22	9	0	4·50	1·40.... 14·02	6·73	0·009**
CL(P)	29	8	2	3·79	1·25.... 11·55	6·35	0·04*
All clefts	51	17	2	4·10	1·50.... 11·18	7·76	0·02*

Homozygosity for C2C2: $\chi^2 = 1·55$; p-value = 0·21.

χ^2 repeated with C1C2 and C2C2 combined in one group:

i) CP: $\chi^2 = 5·23$; p-value = 0·022* * $p < 0·05$

ii) CL(P): $\chi^2 = 5·51$; p-value = 0·019*

iii) All clefts: $\chi^2 = 7·34$; p-value = 0·006** ** $p < 0·01$

Relative Risk for possession of C2 allele with C1C2 and C2C2 combined in one group.

Table 22 Probands genotype frequency

(a) *Bam*HI genotypes.

	A1A1	A1A2	A2A2	χ^2	p-value
Holder *et al.*	0	16	44		
CP	0	2	6	0·01	0·92
CL(P)	0	1	9	1·29	0·25
All Clefts	0	3	15	0·75	0·37

(b) *Rsa*I genotypes.

	B1B1	B1B2	B2B2	χ^2	p-value
Holder *et al.*	10	22	22		
CP	0	4	5	1·79	0·71
CL(P)	0	7	4	3·60	0·16
All Clefts	0	11	9	4·51	0·10

(c) *Taq*I genotypes.

	C1C1	C1C2	C2C2	χ^2	p-value
Holder *et al.*	55	5	0		
CP	5	3	0	5·78	0·016
CL(P)	6	3	0	4·72	0·029
All Clefts	11	6	0	7·86	0·005

* $p < 0·05$

** $p < 0·01$

The parental distribution of alleles was investigated further by subdividing with respect to gender in each of the cleft groups (Table 23). For the combined cleft group there was an increase in maternal frequency for A1 allele (n = 16 from 60 maternal subjects), which just reached the level of statistical significance when compared to the paternal group (n = 5 from 53 paternal subjects) ($\chi^2 = 3.85$, $p = 0.049$). For CP this showed that for the A1 allele there was no statistically significant difference between fathers and mothers, even though the A1A2 genotype was twice as common in the maternal compared to the paternal genotype (n = 10 in mothers and n = 5 in fathers respectively). In the CL(P) group none of the fathers possessed the A1A2 genotype so the A1 allele in CL(P) probands was always transmitted from the maternal genome; and this maternal bias in A1A2 genotype frequency did prove to be statistically significant ($\chi^2 = 5.24$, $p = 0.022$). It also follows from the above that in all cases when the father possessed the A1A2 genotype the child had an isolated CP, but this was only in five of the total sample.

1(b) *Rsa*I genotypes v cleft type. (Table 21(b))

Comparison of all clefts against the control and of CP and CL(P) separately revealed that there were no differences between the *Rsa*I genotype and either cleft category ($\chi^2 = 1.74$, $p = 0.42$ for CP and $\chi^2 = 0.78$, $p = 0.68$ for CL(P).

The proband results for the *Rsa*I genotypes likewise revealed no significant differences between cleft and control genotype frequencies for either CP or CL(P) (Table 22(b)).

1(c) *Taq*I genotypes v cleft type. (Table 21(c))

Digestion of the TGFα with *Taq*I restriction enzyme reveals a predominance of the C1 allele relative to the C2 polymorphism, the C2 allele having a prevalence of only 4.2% in this control population. As with the *Bam*HI A1 polymorphism, homozygosity for the C2 allele is rare, and in fact only two C2C2 cases presented among the parents, both being fathers of children with CL(P).

Table 23(a) Parental distribution of alleles.

A1/A2 polymorphism.

	Paternal	Maternal	Relative Risk (R)		χ^2	p-value
	A1/A2	A1/A2	A1	95% CI		
CP	5/21	10/26	1·62	0·48 ..... 5·45	0·60	0·44
CL(P)	0/32	6/34	12·25	1·18 .. 127·62	5·24	0·022*
All	5/53	16/60	2·83	1·06 ..... 7·98	3·85	0·049*

Table 23(b) Parental distribution of alleles.

C1/C2 polymorphism.

	Paternal	Maternal	χ^2	p-value
	C1/C2	C1/C2		
CP	4/22	5/31	0·027	0·869
CL(P)	4/31	5/37	0·004	0·948
All	8/53	10/68	0·002	0·959

* $p < 0·05$

Analysis of the three genotype frequencies for all parents v controls using the chi-squared test statistic with two degrees of freedom allowed the null hypothesis to be rejected in favour of the alternative for the *Taq*I genotypes (χ^2 = 7.76, p = 0.02) suggesting that a real difference does exist in the TGFα genotypes at the *Taq*I determined locus, between parents of a child with clefting and a control. This test has expected frequencies of two for the C2C2 allele however, which inflates the chi-squared test statistic and reduces the validity of the result obtained. To overcome the inflated test statistic the C1C2 and C2C2 genotypes were combined and the test re-applied with one degree of freedom (χ^2 = 7.34, p = 0.006). This result confirms the difference between controls and parents, suggesting that a parent with the C1C2 or C2C2 genotype has an increased likelihood of having a child with clefting.

To test if there was any association between genotype and the type of cleft defect, the chi-squared test was repeated comparing the CP and CL(P) figures against the control. The result of this indicates that the increased prevalence of the C1C2 or C2C2 genotype is significant in only the CP parents (χ^2 = 6.73,p = 0.009) and just reached the 5% significance level in the CL(P) group (χ^2 = 6.35, p = 0.04).

Homozygosity for the C2 allele did not feature among the probands in this study, but the frequency of the C1C2 genotype was increased in both CP and CL(P) proband groups (p = 0.016 for CP and p = 0.029 for CL(P)) (Table 22(c)). The small numbers in the latter study, however, renders the result meaningless, and a larger proband sample for both cleft types would be required to validate these findings.

Finally, to check for maternal/paternal effects in the *Taq*I generated genotype distribution, the frequency in mothers and fathers was compared (Table 23(b)). This shows no signs of maternal or paternal bias in the distribution of either the C1C1 or C1C2 genotypes. It was also noted that the only two instances of homozygosity for the C2 allele were in fathers of children with unilateral cleft lip and palate, and that these two individuals had the same haplotype (A2A2, B2B2, C2C2) with regard to TGFα. It would be incorrect however to draw conclusions, or even speculate on the strength of a sample of two.

To summarise the results at this stage, it could be concluded that to estimate a particular individuals likelihood of producing a child with CP or CL(P) digestion of a DNA sample with *Taq*I could be carried out. Presence of the C2 allele, and either the C1C2 or C2C2 genotype would indicate predisposition to clefting, but is not a sensitive discriminator between CP and CL(P). *Bam*HI digestion, however, showing the presence of the A1A2 genotype in either parent would be indicative of genetic predisposition to isolated CP.

3.2.2 Relative risk analysis

The relative risk analysis (R) was applied to all of the parental allele frequency data and those instances where a significant difference in genotype frequency was found when comparing the parental and control data. It is merely a quantitative measure of the probability of finding a particular allele or genotype in one group relative to the other.

i) Parental allele frequency:

Although the figures did not reach statistical significance using the chi-squared test the probability of finding the A1 allele in the CP parental group relative to the control was increased by a factor of 2·17 and reduced in CL(P) parents to 0·57. Furthermore the probability of finding the A1 allele in the maternal genome relative to that of the father of a child with CP is increased by a factor of 1·62, but for CL(P) the chance of finding the A1 allele on the maternal genome is increased by a factor of 12·25. The latter result is inflated, however by the fact that the figure for the paternal A1 allele was zero.

For the C2 allele there is an increased probability of it's presence in the genome of the parents of both CP and CL(P) probands, by a factor of 3·91 and 3·38 respectively. No maternal or paternal effect was apparent in the *Taq*I RFLP alleles. The equivalent relative risk figures for the C2 allele in CP and CL(P) probands was 5·31 and 4·60, albeit in small numbers of subjects.

ii) Parental genotype frequency:

There was an increased probability of a CP parent possessing the A1A2 genotype, by a factor of 2·75 relative to the control while a CL(P) parent was less likely to possess the A1A2 genotype (R = 0·53). The probability of finding the C2 allele in either the

heterozygous of homozygous form in the parental genotypes was 4·50 for CP and 3·79 for CL(P).

3.2.3 Logistic regression using genotype data.

The first logistic regression model to determine the relationship between genotype and liability to clefting (either CP or CL(P)) was set up as follows:

Response - "cleft parents" v control

Factor 1 - *Bam*HI (A1A2 or A2A2)

Factor 2 - *Rsa*I (B1B1 or B1B2 or B2B2)

Factor 3 - *Taq*I (C1C1 or C1C2/C2C2)

A forward stepwise approach was adopted and a lattice of hypotheses was produced. The effect of *Taq* C2 allele was entered first (as C1C2 or C2C2 genotype). None of the other factors, or possible interaction terms, (Factors 1-3 above) was entered afterwards. This simply means that liability to clefting is determined by the *Taq*I C2 allele alone. The resulting logistic regression is effectively equivalent to a simple Chi-squared test. The latter test had indicated that the C2 allele predisposed to clefting ($\chi^2 = 6.82$, $p = 0.0009$) in an earlier analysis (see Table 19(c)).

A second logistic regression model was set up to determine which of the following four factors would best discriminate between a parent predisposed towards producing a CP and CL(P) child. The factors used were the TGFα genotypes produced by *Bam*HI, *Rsa*I and *Taq*I restriction enzyme digestion, plus parental gender. The resulting logistic regression model indicates that the best possible genotype discriminator between CP and CL(P) parents is a *Bam*HI/*Rsa*I interaction. The effect of this interaction on the outcome of the child's birth defect is illustrated by the cross classification of the respective genotypes produced by *Bam*HI and *Rsa*I restrictive enzyme digestion for both CP and CL(P) (Table 24). For parental genotype A1A2/B1B2, the outcome is predominately a cleft palate child, while A2A2/B1B2 predisposes to CL(P). Using the present model with these genotypic markers, 68.3% of parents were correctly classified according to the child's birth defect (CP or CL(P)).

Table 24 Genotype/Cleft type interaction.

A:CLEFT PALATE

		*Rsa*I			
		B1B1	B1B2	B2B2	ALL
	A1A2	0	12	1	13
*Bam*HI	A2A2	2	2	11	15
	ALL	2	14	12	28

B:CLEFT LIP AND PALATE

		*Rsa*I			
		B1B1	B1B2	B2B2	ALL
	A1A2	0	4	1	5
*Bam*HI	A2A2	3	13	14	30
	ALL	3	17	15	35

This analysis of the genotypes resulting from restriction enzyme digestion by *Bam*HI, *Rsa*I and *Taq*I reveals the evidence of linkage disequilibrium at the TGFα site between *Bam*HI and *Rsa*I polymorphisms (Table 24). This disequilibrium differentiates between parents of CP and CL(P) children.

Further analysis of the *Bam*HI/*Rsa*I genotype interaction was carried out using a chi-squared test on the number of subjects presenting with A1A2/B1B2 and A2A2/B2B2 in the CP, CL(P) and control groups (Table 25). This revealed a significant association between A1A2/B1B2 and CP (χ^2 = 4·30, p = 0·038). The association between A2A2/B1B2 and CL(P) compared to the control did not reach the 5% level of significance (χ^2 = 3·34, p = 0·067). There was however a highly significant difference in the *Bam*HI/*Rsa*I genotype interaction when comparing the two cleft groups (χ^2 = 11·89, p = 0·0006), emphasising the potency of these genotypes in the discrimination between a parents liability towards producing a child with CP or a child with CL(P).

Table 25 *Bam*HI/*Rsa*I genotype interaction for CP, CL(P) and control groups.

Genotype	B1B2		
	CP	CL(P)	Control
A1A2	12	4	12
A2A2	2	13	11
*Bam*HI/*Rsa*I interaction	χ^2	p-value	
CP v control	4.30	0.038*	
CL(P) v control	3.34	0.067	
CP v CL(P)	11.89	0.0006***	

* = $p < 0.05$

*** = $p < 0.001$

3.3 ANALYSIS OF PHENOTYPIC/GENOTYPIC DATA

3.3.1 Parental genotype/phenotype comparison.

Analysis of variance was used to determine whether any relationship existed between parental genotype and their craniofacial measurements, carrying out the analysis for mothers and fathers separately. Separate analysis of males and females was necessary because of the fact that the previous cephalometric study has shown that definite gender differences in craniofacial parameters do exist; and combining the data might well mask a significant gender effect in this genotype/phenotype analysis. The cephalometric data was adjusted for age and skeletal class as was done prior to the discriminant analysis earlier in the study.

Each of the 67 craniofacial parameters was assessed in turn for the *Bam*HI, *Rsa*I and *Taq*I generated TGFα polymorphisms for both fathers and mothers, and the results displayed in a total of 402 boxplots. Genotype was plotted against each parameter with CP and CL(P) identified separately. Samples of these are provided to illustrate the results (see Figures 39 (a-f) for males and Figures 40 (a-f) for females).

This study has identified significant differences in parental craniofacial morphology when compared to a control and differences are apparent between the different cleft types within the parents. In addition DNA from parents shows a significant increase in the frequency of certain alleles in the region of the TGFα locus.

Further analysis of the data from this study was undertaken to investigate whether any relationship existed between the genotype of the parents and their craniofacial morphology; and also whether parental information can be used to predict a child's birth defect, i.e. are there phenotypic or genotypic markers for clefting in the parents. No previous study has used this type of combined phenotype/genotype approach in the investigation of orofacial clefting. The gender differences highlighted in both the genetic and the morphological studies can simultaneously be investigated in this analysis.

For each craniofacial variable in turn plotted against a particular genotype (which occurred in varying frequencies among the parents) analysis of variance tables and box

plots for cleft palate and cleft lip and palate were produced (Figures 39 and 40). Results for male and female parents are presented separately below.

a(i) Fathers: *Bam*HI genotype v craniofacial parameters

The A1 allele is relatively rare and, in fact, the A1A1 genotype was unrepresented in either fathers or mothers in this sample and furthermore A1A2 was unrepresented in CL(P) fathers. As a result only three genotypes are represented in Figures 39(a) and 39(b).

In only one of the 67 parameters, the orbital prominence measurement (S-N-Or), was there a difference between the A1A2 (n = 5) and A2A2 (n = 24) genotypes, this being larger in the A2A2 fathers (Figure 39(a)). For all other parameters no significant difference due to the *Bam*HI determined genotype was apparent. Figure 39(b) is a typical example.

a(ii) Mothers: *Bam*HI v craniofacial parameters.

Only one craniofacial measurement, the sella-nasion line to Frankfort plane angle (SN-FP) was shown to have a linear relationship with maternal genotype. In mothers homozygous for the A2 allele, A2A2 (n = 22), this angle was greater than in those with A1A2 (n = 16) (Figure 40(a)). None of the other 66 craniofacial measurements showed any significant association with genotype, and plots such as that shown in Figure 40(b) were typical.

b(i) Fathers: *Rsa*I genotype v craniofacial parameters.

Homozygosity for the B1 allele presented in only two male parents and therefore is of little value in determining an association. Fathers homozygous for the B2 allele (i.e., B2B2, n = 12) tended towards a larger upper face height (N-ANS) (Figure 39(c)), but smaller cranial parameters (occipital chord, parietal chord and total cranial area) than fathers possessing the B1B2 genotype (n = 16). This was not a consistent trend, however and could readily be explained by chance, as the cranial parameters are likely to be correlated. Figure 39(d) is a plot of the variation of mandibular area with genotype showing no apparent association and this was a more typical pattern.

b(ii) Mothers: *Rsa*I genotype v craniofacial parameters

The B1B1 genotype was also relatively rare in mothers of cleft children, being present in only one CP mother and three CL(P) mothers which reduced its discriminative power. In any case there was no apparent association between maternal craniofacial measurements and *Rsa*I determined genotype. Figure 40(c) shows one parameter (ramus width) which would appear to show a trend towards increasing width according to genotype (B1B1 > B1B2 > B2B2), but this failed to reach significance, and the relationship between mandibular area and genotype was much more typical (Figure 40(d)).

c(i) Fathers: *Taq*I genotype v craniofacial parameters

On TGFα digestion with *Taq*I the C2 allele was relatively rare and in the CP fathers homozygosity for C2 did not present, while only two CL(P) fathers had C2C2. Careful scrutiny of all the genotype versus cephalometric parameter plots indicated that only three appeared to show a trend. A representative example of this was the Se-PNS measurement (posterior face height) which appeared to be reduced in those subjects with the C2 allele (Figure 39(e)). None of the other 64 parameters showed any consistent trend and Figure 39(f) is a typical example.

c(ii) Mothers: *Taq*I genotype v craniofacial parameters

In the maternal genotypes for *Taq*I, none were homozygous for the C2 allele, and therefore the comparison was limited to C1C1 (n = 29) and C1C2 (n = 10) mothers in both cleft categories. Again only a few parameters such as nasal area (Figure 40(e)) showed any association with the *Taq*I genotype, the C1C2 mothers being larger than their C1C1 counterparts. Area of mandible (Figure 40(f)) serves to illustrate the more typical trend for 63 of the 67 maternal craniofacial parameters plotted against genotype.

To summarise, the parental genotype/phenotype cross-correlation shows that in only the exceptional instance was there any trend between a particular craniofacial measurement and genotype; and with all 67 parameters being included in the analysis there is a statistical likelihood that this would happen merely by chance. It can therefore be concluded that there is no significant overall relationship between genotype and parental craniofacial size or shape as assessed from lateral cephalograms.

The conclusion from the above results is that no association was found between the polymorphic alleles produced by restriction digestion with *Bam*HI, *Rsa*I or *Taq*I at the TGFα site and parental craniofacial morphology.

3.3.2 Role of Phenotype/Genotype interaction in prediction of child's birth defect

The analysis of variance carried out earlier highlighted the association between certain parental craniofacial variables namely cranial area (c6), mandibular area (s2) MMPA (s35) and mandibular ramus length (Cd-14) and the type of birth defect in the child (see Table 15). The incorporation of this phenotypic data into the previous model may further improve the ability to predict outcome in terms of type of birth defect. A logistic regression model already containing the *Bam*HI and *Rsa*I genotypes main effects and their interaction was therefore addressed with the parental craniofacial data. The same 37 cephalometric variables used in the stepwise discriminant analysis were chosen and these were adjusted for gender and skeletal class for this logistic regression analysis. Four of them were significant and these were therefore incorporated into the model: total facial length (S-Gn), maxillary to mandibular planes angle (MMPA), anterior cranial base length (S-N) and cranial width (Gla-CPo).

Another lattice of hypotheses model was produced which allows the simple model including the main effects of these four craniofacial measurements to be considered alongside more complex models that include their interactions with genotypic data. With the *Bam*HI and *Rsa*I interaction, plus the four above-mentioned craniofacial variables, 19 out of 25 CP parents (76%) and 30 out of 32 CL(P) parents (94%) were correctly classified. This is an overall rate of 86% correct.

These data were also analysed without forcing the genetic variables into the model and the same four cephalometric variables were entered in the same order. This resulted in a reduction in discrimination for CP with 18 out of 25 parents (72%) being correctly classified, and a more significant reduction for CL(P) with 25 out of 32 CL(P) parents (78%) being 75% overall. The use of the genetic information clearly improves the ability to discriminate over using the craniofacial measurements alone.

The four craniofacial parameters chosen by this model may not be the only significant discriminators. Other parameters may be masked by their association with either *Bam*HI or *Rsa*I genotypes already incorporated in the model. An example is cranial area (c6) shown in this study to be a potent discriminator between CP and CL(P), the effect of which is taken up by the *Bam*HI genotype discrimination. (i.e. irrespective of genotype A1A1 or A2A2 in the group, a larger cranial area (c6) measurement was found to predispose to isolated CP in this study).

Figure 39: (a) - (f)

Box and Whisker Plots for Selected Paternal Craniofacial Variables Plotted Against Genotype and Labelled for Cleft Type

Key

CP

CL (P)

A, B, C - Labelling of genotypes according to restriction enzyme digestion with *Bam HI*, *Rsa I* and *Taq I* respectively

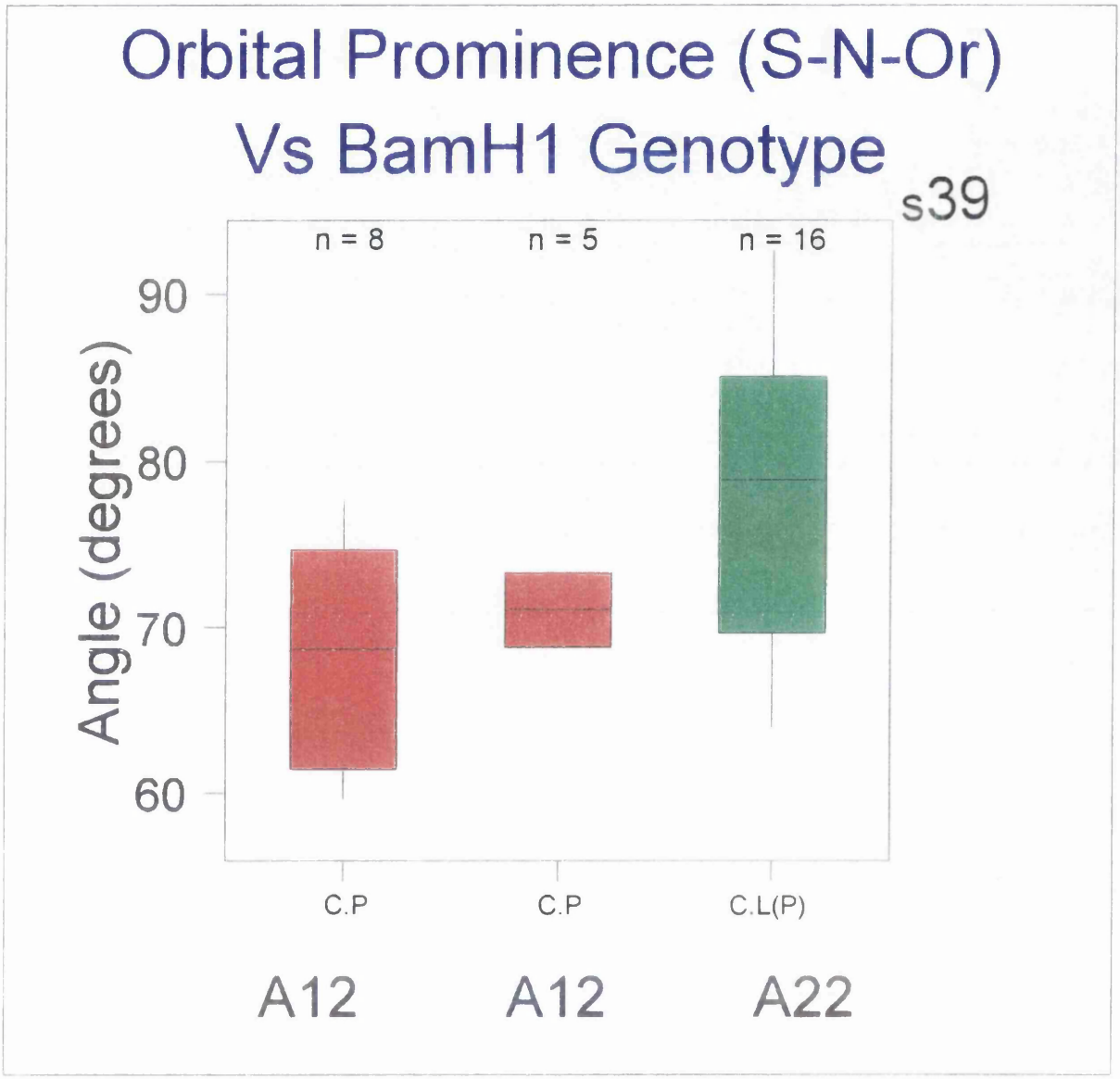


Figure 39 (a) Box and whisker plot showing variation between orbital prominence (S - N - Or) and Bam HI genotype for fathers in both cleft groups

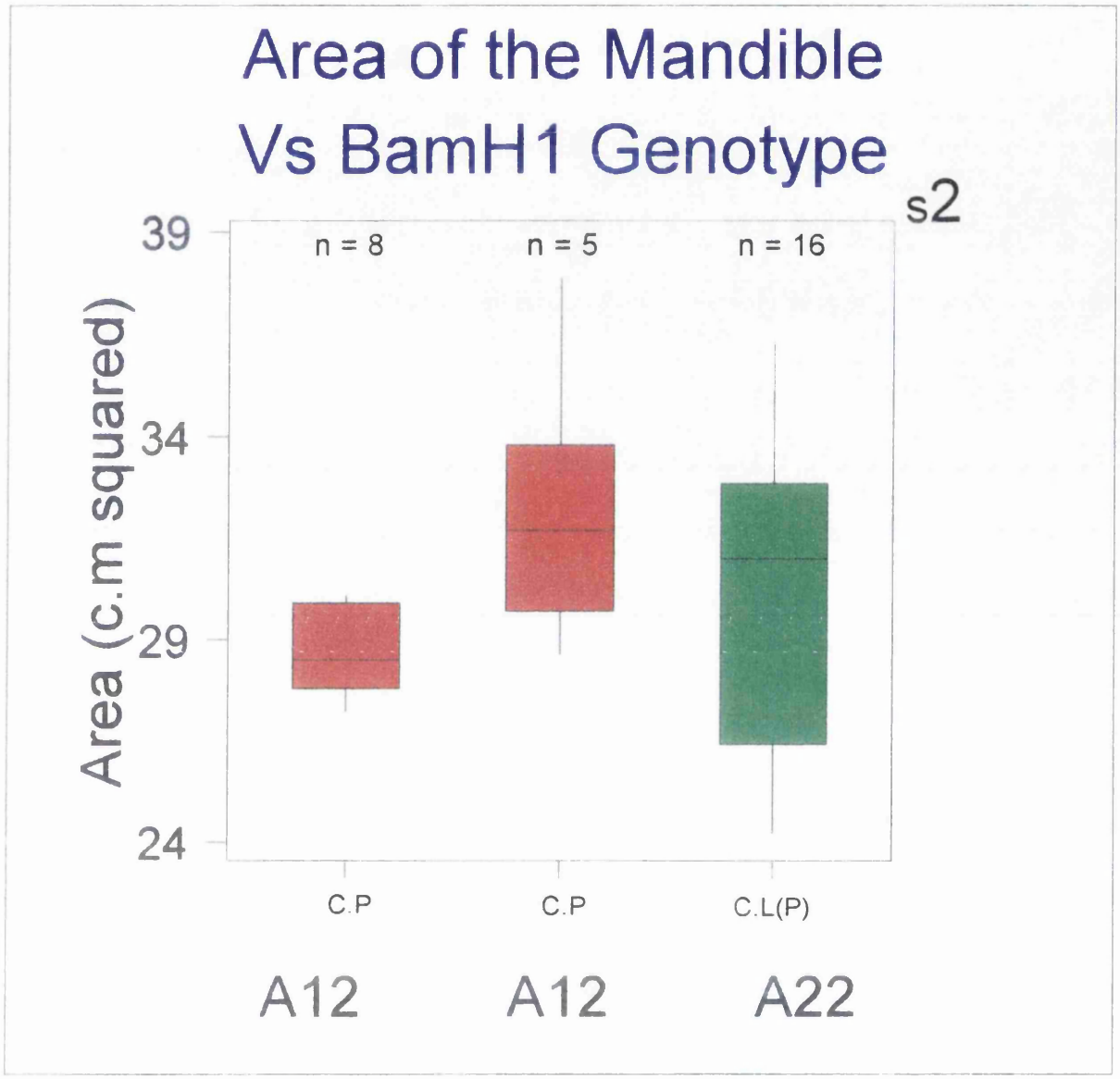


Figure 39 (b) Box and whisker plot showing variation between area of the mandible and Bam HI genotype for fathers in both cleft groups

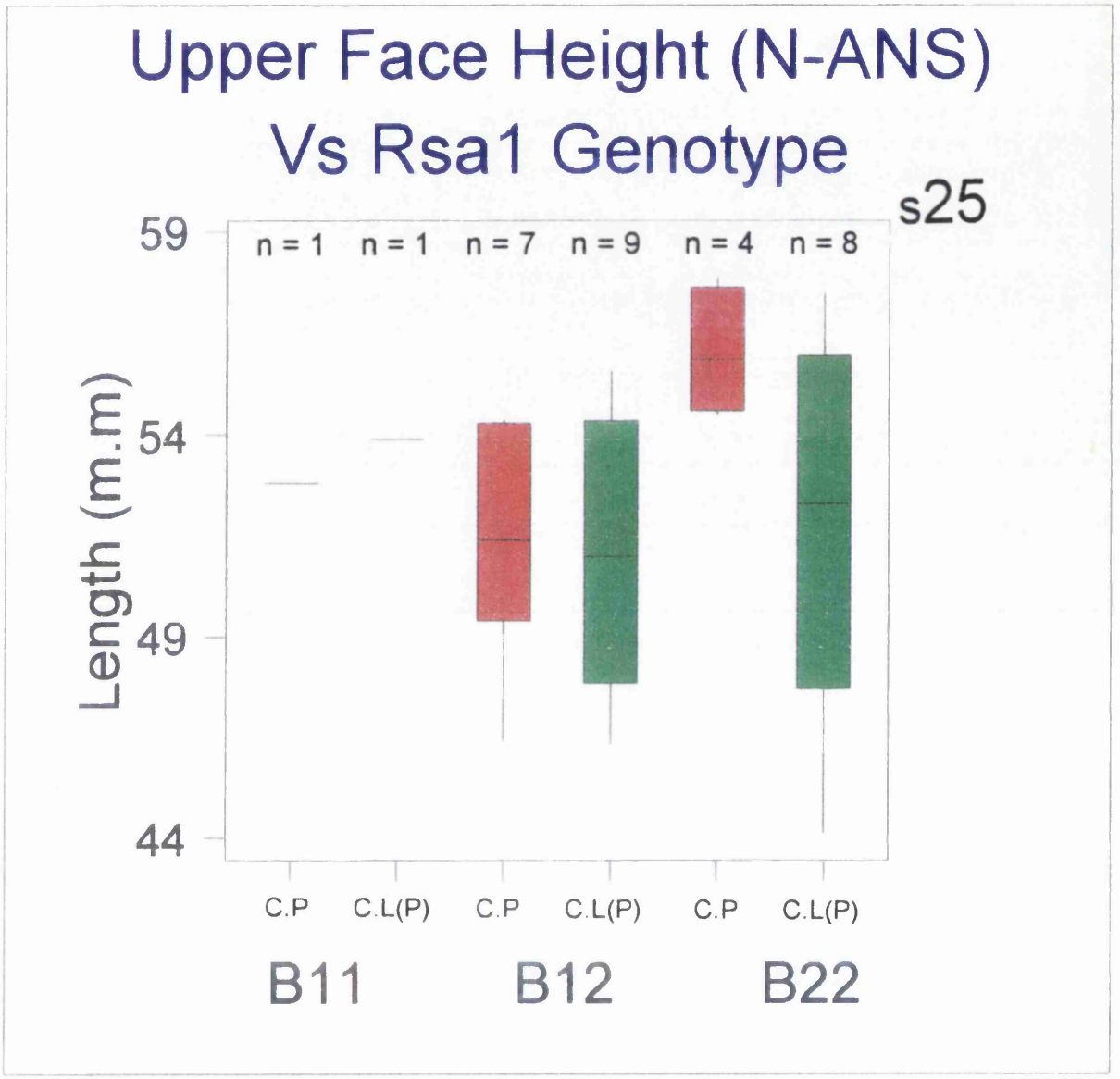


Figure 39 (c) Box and whisker plot showing variation between upper face height (N -ANS) and RSA I genotype for fathers in both cleft groups

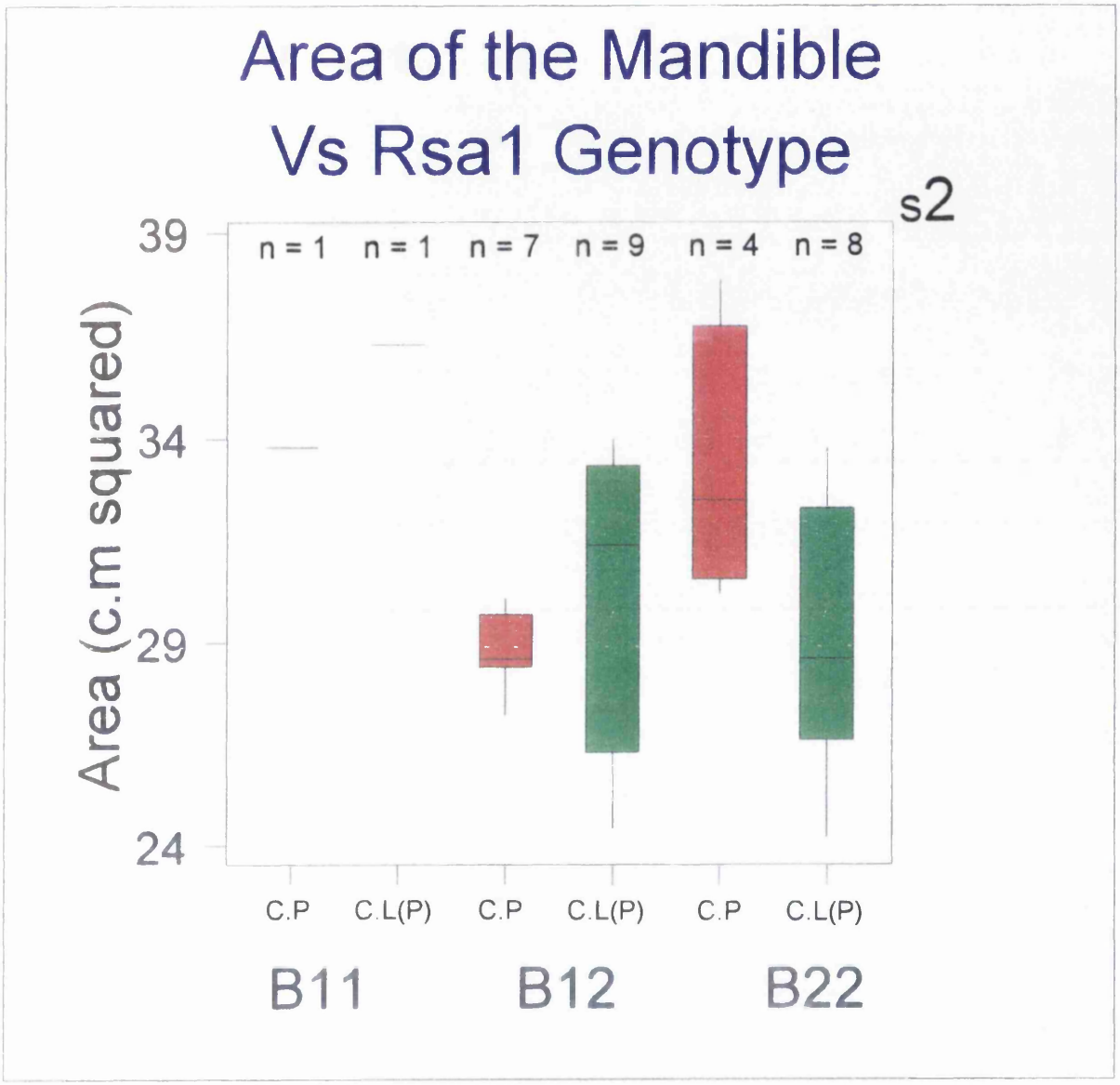


Figure 39 (d) Box and whisker plot showing variation between area of the mandible and Rsa I genotype for fathers in both cleft groups

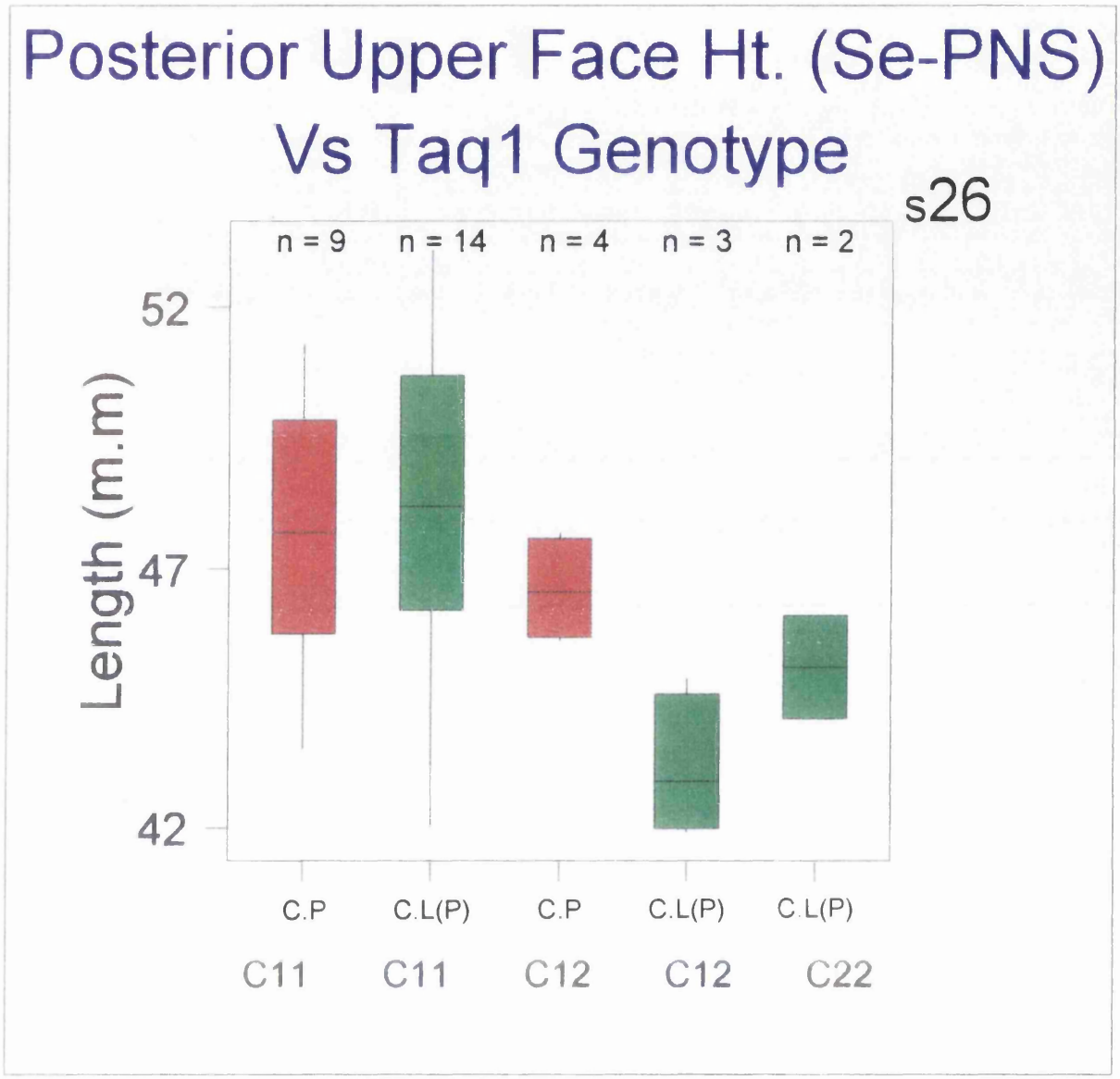


Figure 39 (e) Box and whisker plot showing variation between posterior upper face height (Se - PNS) and Taq I genotype for fathers in both cleft groups

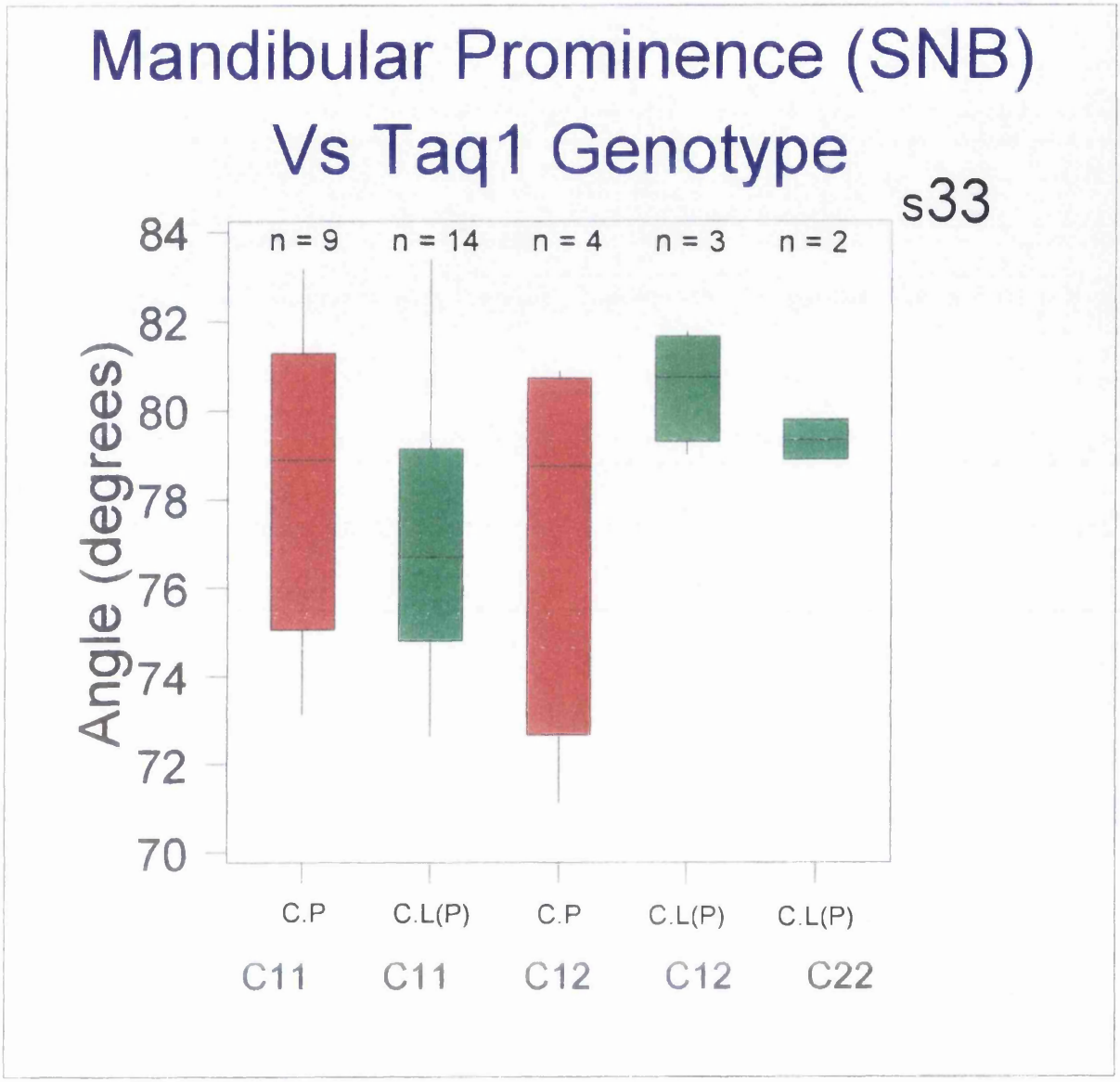


Figure 39 (f) Box and whisker plot showing variation between mandibular prominence (SNB) and Taq I genotype for fathers in both cleft groups

Figure 40: (a) - (f)

Box and Whisker Plots for Selected Maternal Craniofacial Variables Plotted Against Genotype and Labelled for Cleft Type

Key

CP

CL (P)

A, B, C - Labelling of genotypes according to restriction enzyme digestion with *Bam HI*, *Rsa I* and *Taq I* respectively

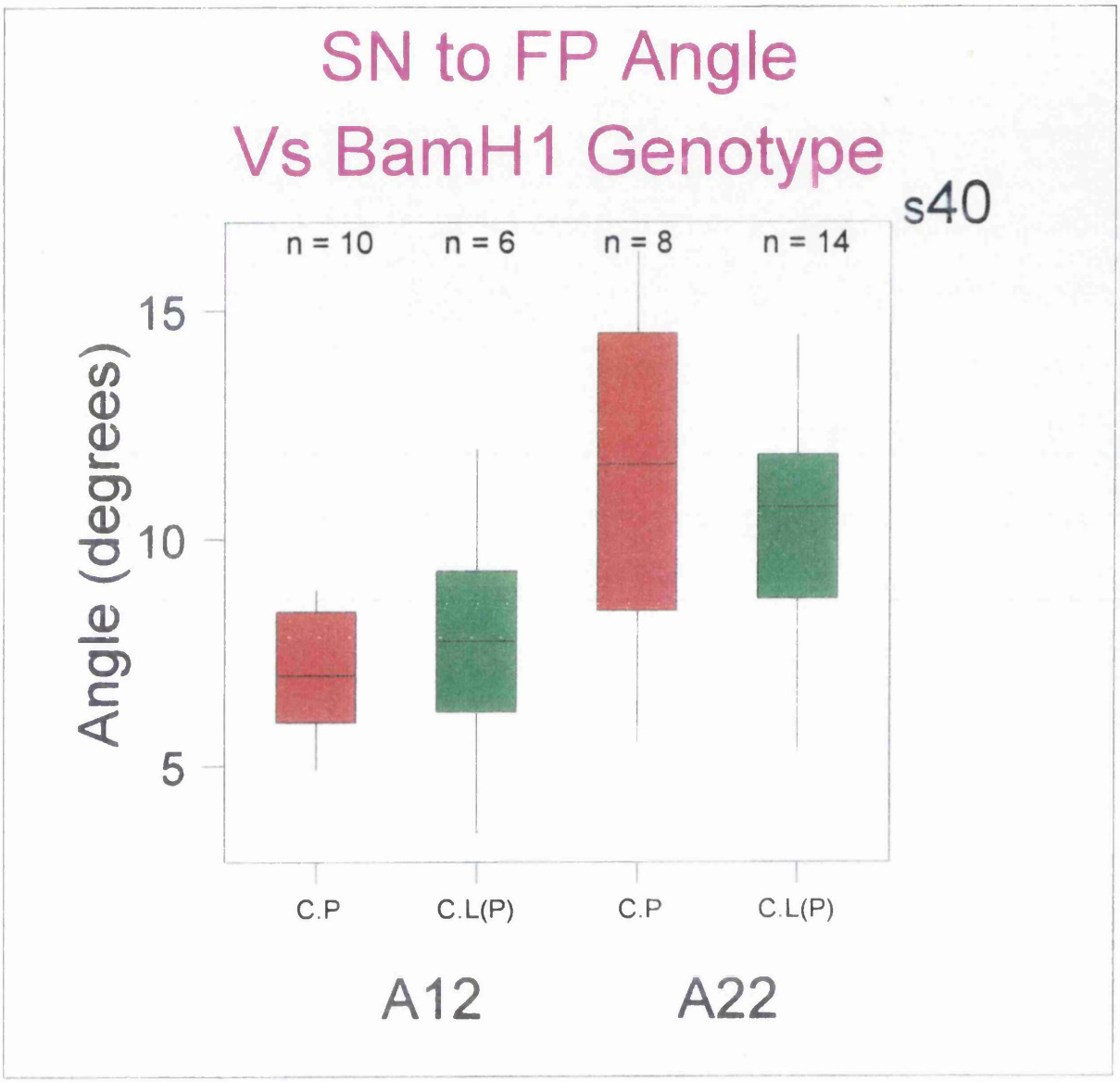


Figure 40 (a) Box and whisker plot showing variation between SN to FP angle and Bam HI genotype for mothers in both cleft groups

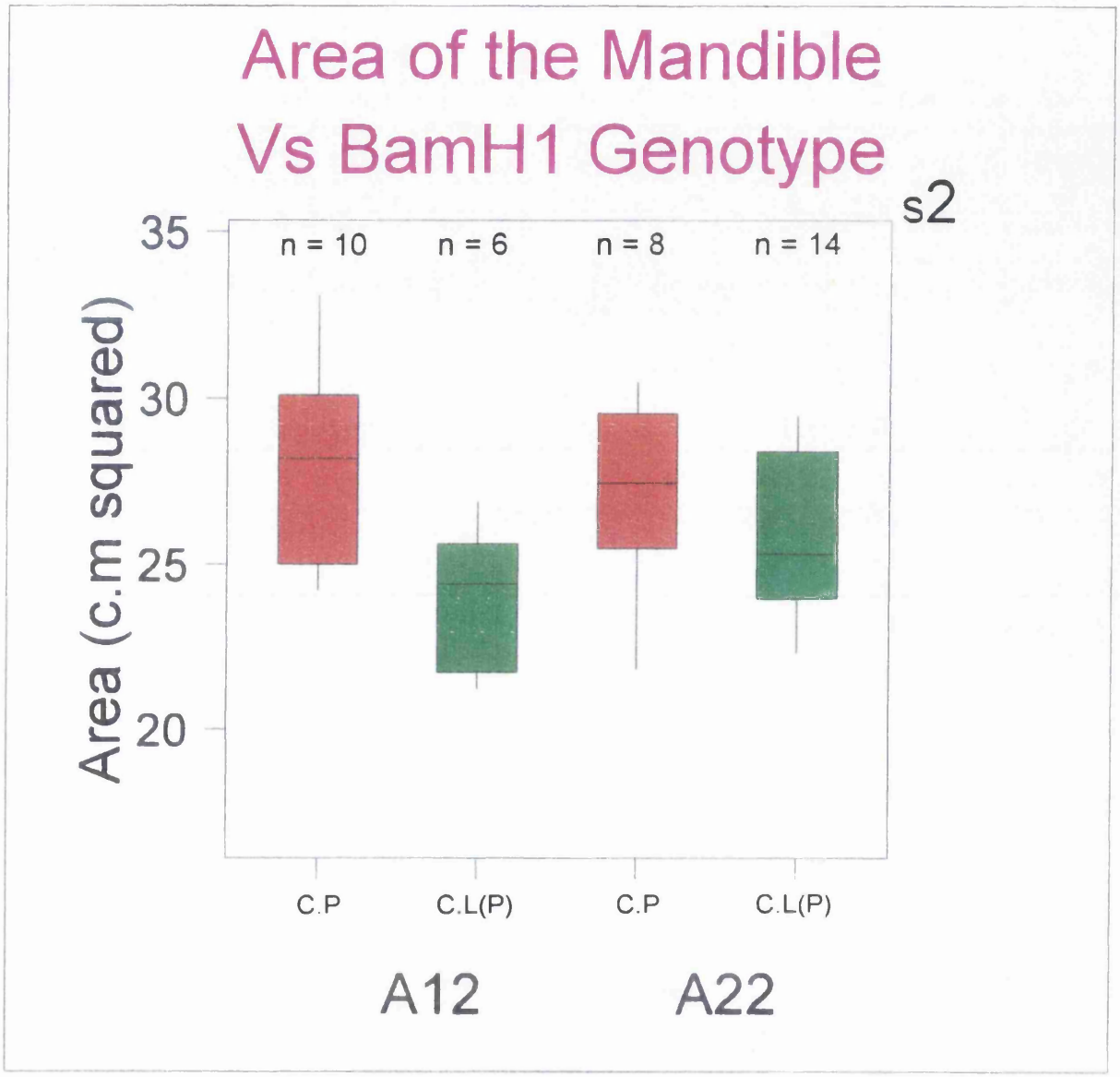


Figure 40 (b) Box and whisker plot showing variation between area of the mandible and Bam HI genotype for mothers in both cleft groups

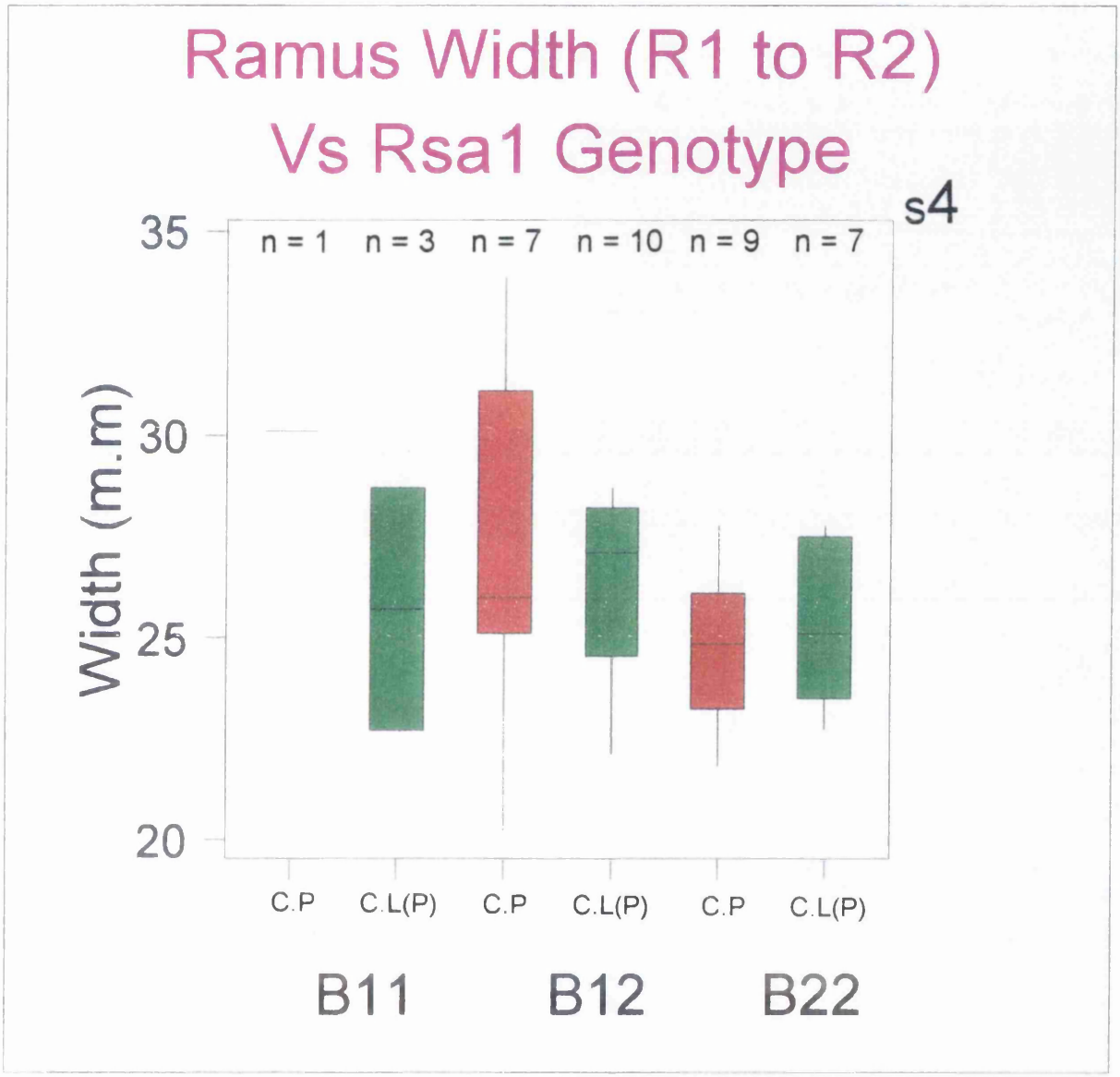


Figure 40 (c) Box and whisker plot showing variation between ramus width (R1 to R2) and RSA I genotype for mothers in both cleft groups

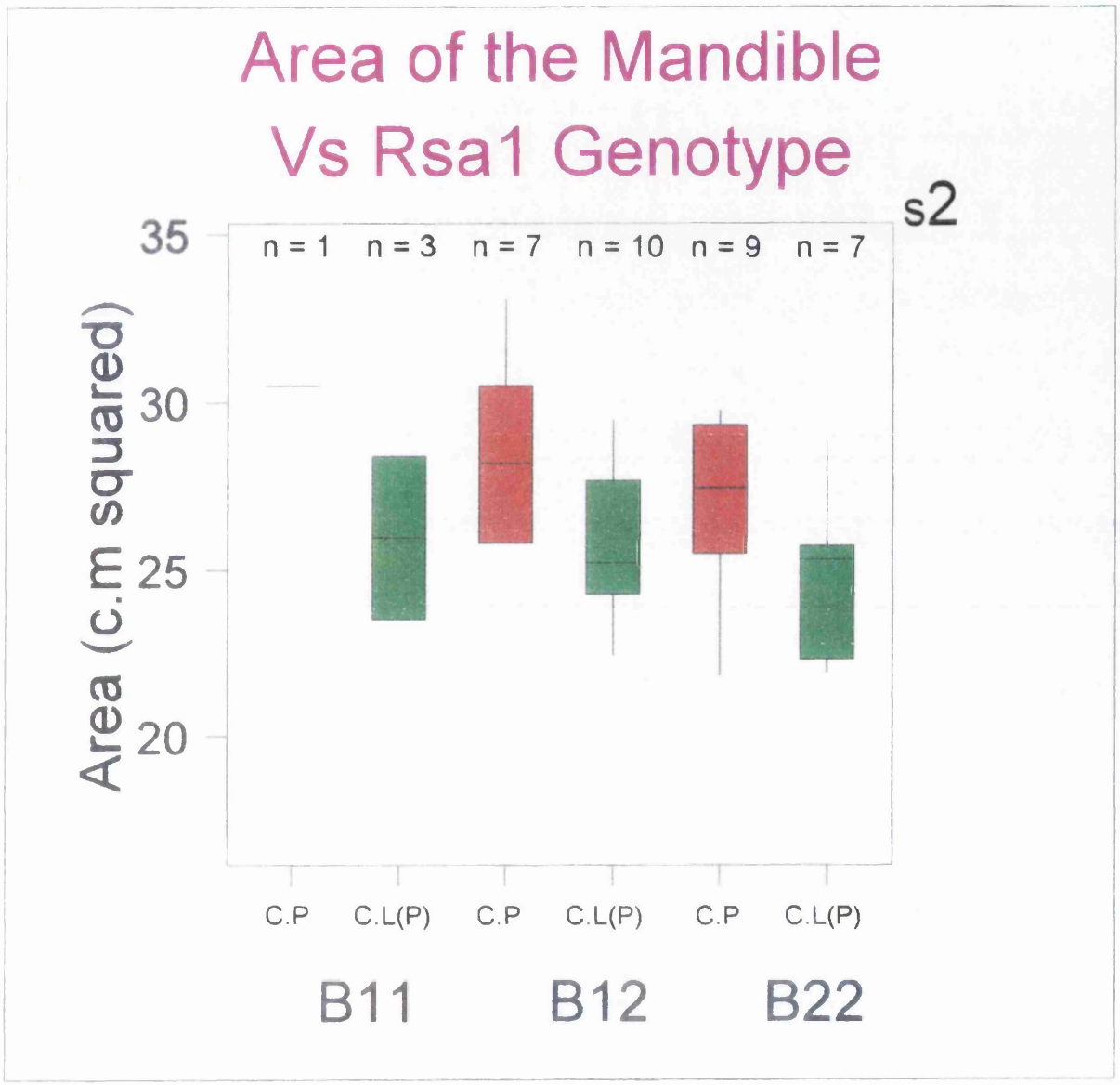


Figure 40 (d) Box and whisker plot showing variation between area of the mandible and Rsa I genotype for mothers in both cleft groups

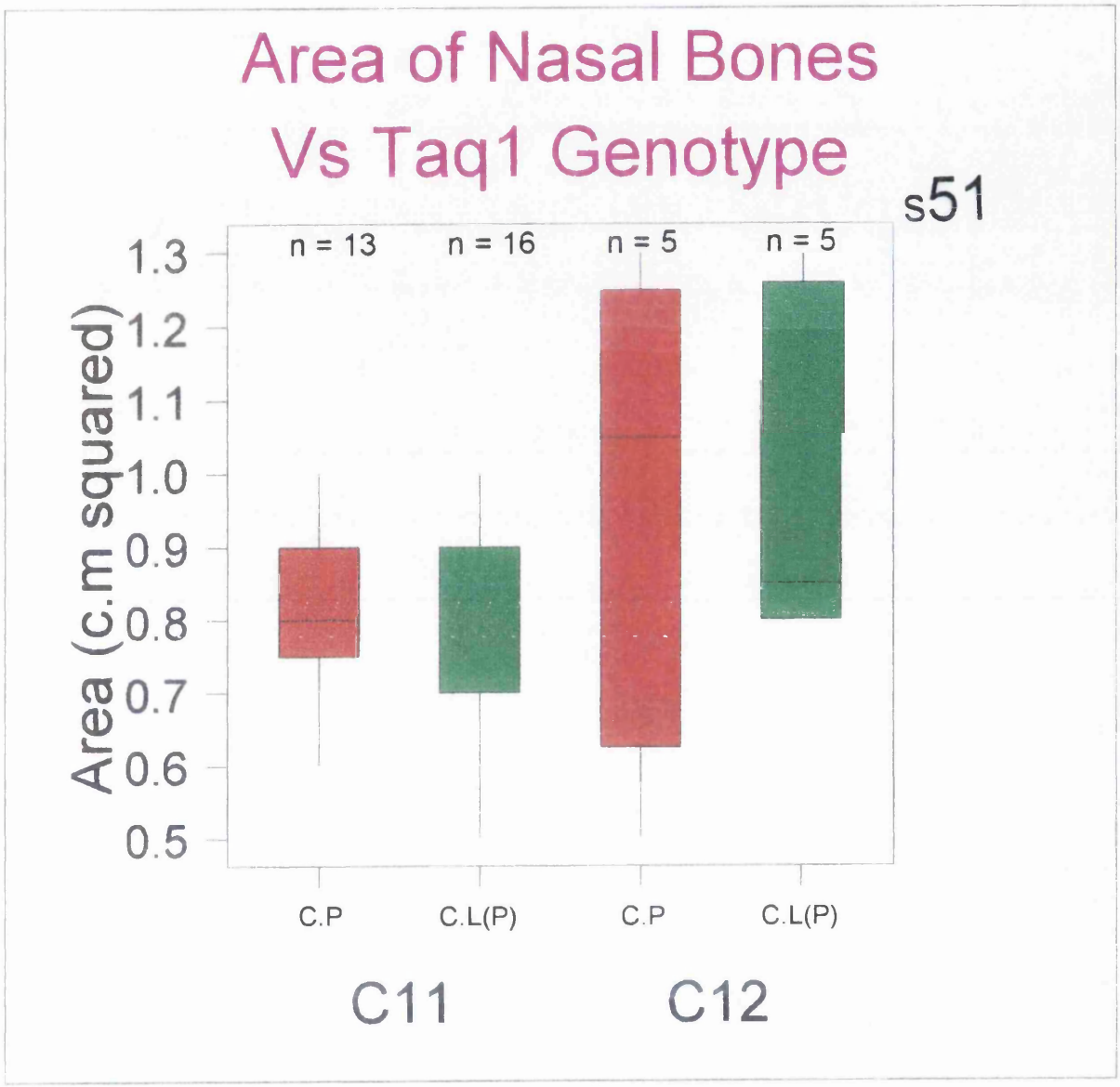


Figure 40 (e) Box and whisker plot showing variation between area of nasal bones and Taq I genotype for mothers in both cleft groups

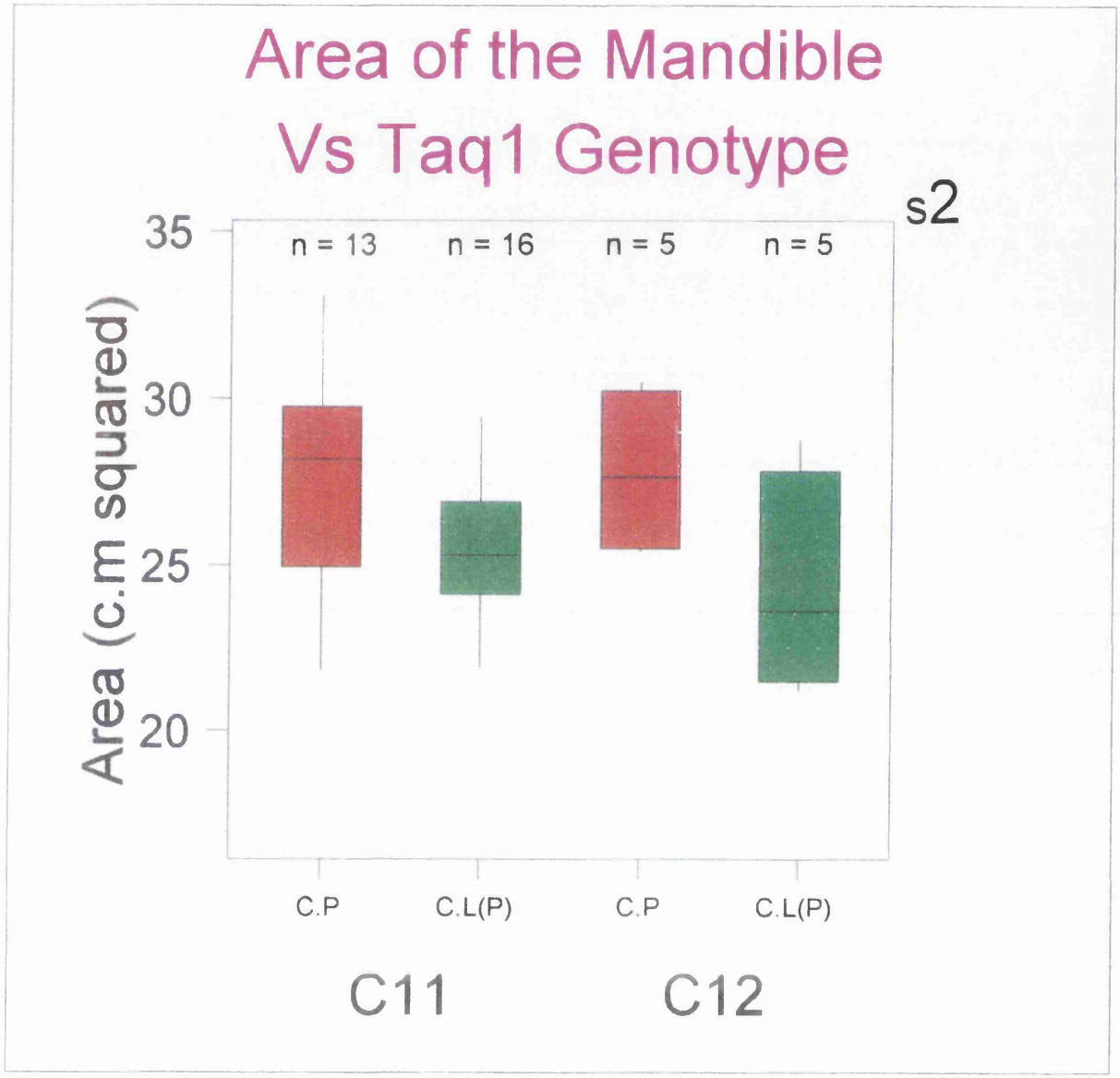


Figure 40 (f) Box and whisker plot showing variation between area of the mandible and Taq I genotype for mothers in both cleft groups

CHAPTER 4

DISCUSSION

4.1 CEPHALOMETRIC RESULTS

The results of the present cephalometric study are consistent with all those carried out previously in that craniofacial morphology of parents of children with CP or CL(P) is distinctly different to that of parents of children without clefts. There is however little consensus on which parameters are significantly different. Table 26 records the results of ten previous studies investigating these differences and apart from the reduction in upper face height none of the more commonly used parameters consistently studied show similar differences. This may be explained, at least in part, by the lack of uniformity in study designs, sample sizes, male to female ratios and varying proportion of different types of cleft subjects involved. The study by Ward *et al.* (1989) added a new dimension to the study of craniofacial morphology as a risk indicator in the context of predisposition to cleft lip and palate. They highlighted the lack of concensus in previous studies and offered a credible explanation, that all of these had grouped fathers and mothers with the tacit acceptance of the multifactorial threshold model for the transmission of clefting anomalies. Many had also included both CL(P) and CP parents in their study samples and analysed them together. Evidence is accumulating for the influence of a major gene or genes in the aetiology of clefting, and it may well be that one parent may contribute more to the susceptibility for oral clefting than the other. Ward *et al.* (1989) used a multivariate method of statistical analysis known as a cluster analysis. This is a method of numerical taxonomy which allows grouping of individuals with the most characteristics in common, provided these characteristics are expressed numerically. This method lends itself well to conventional craniofacial cephalometrics as the use of computer technology allows pattern recognition from large sets of numerical data. Application of this technology to the cephalometric data from parents of children with cleft lip and palate enabled Ward *et al. (1989)* to categorise the data into groups in which the association is high among members of the same group; the aim being to search for a unique set of cephalometric features, and the hypothesis being that only one of a "parental pair" need possess the craniofacial morphological features which predispose to cleft lip and palate in their offspring. Three major clusters comprising 17, 39 and 12 individuals emerged from the study, for which mean pattern profiles were produced. The largest of the three clusters (48% of the total sample) showed the least pronounced deviation from the normal mean values and was considered to consist of individuals with "normal" craniofacial morphology.

Table 26 Previous parental cephalometric studies:
Significant craniofacial variables compared to their respective controls

*	1	2	3	4	5	6	7	8	9	10	11
Mandible											
LFH					-	+ ♂	+	+			
Gonial ∠			-	-		+				+	
Ramus					+						+ ♀
Body						-					
SNB											
Maxilla											
UFH		-		-	-		+		-	-	
ANS-PNS		-			-	+		+		+	- ♂
PFH					-	+ ♀		-			
SNA	-									+	
Facial											
TFH	+					+	+		-	-	+ ♀
ANB	-	-	-	-		+		-			
MMPA											
Cr Base											
S-N						+					+ ♀
S-Ba											+ ♀
N-S-Ba		-		+	+			-		+	- ♂
Cranium											
Length					-				-		
Area					-						-

*Previous studies - (see Table 2 for details)

1. Fraser and Pashayan 1970
2. Coccaro *et al.*, 1972
3. Kurisu *et al.*, 1974
4. Shibasaki and Ohtsuka 1978
5. Nakasima and Ichinose 1983
6. Prochazkova and Tolarova 1986
7. Sato. 1989
8. Ward *et al.*, 1989
9. Blanco *et al.*, 1992
10. Raghaven *et al.*, 1994
11. Present study

Key

♀ = Females only
♂ = Males only

- = reduced
+ = increased

Ward *et al.* (1989) concluded from the evidence of his study that many sporadic CL(P) patients may have a genetic component that is indeed derived from one of the parents. A follow up study indicated that individuals with phenotypic features that predisposed them to produce children with clefts showed many similarities when compared to such a cleft group. The one factor that the Ward study did not investigate was that of gender i.e. the relative proportions of mothers and fathers in the "cleft susceptible" groups. In the context of predisposition to clefting the present study is the only parental cephalometric study to segregate subjects on the basis of cleft type and gender simultaneously.

Ward *et al.* in the above mentioned study reported that only one pair of parents presents in each of the "at risk" phenotype clusters. In view of the speculation that there may be a maternal bias in the contribution to clefting it would have been interesting to have analysed the gender ratio in each of these "at risk" clusters. Also studies by Prochazkova and Tolarova (1986) and Figalova *et al.* (1974) reported definite sex differences in parental craniofacial morphology when compared to a control group and Blanco *et al.* (1992) comparing affected CL(P) subjects with their relatives found that certain parameters differed in females only. Since evidence for the influence of a small number of major genes is accumulating, (Marazita *et al.*, 1984, 1986a; Chung *et al.*, 1986,1989; Hecht *et al.*, 1991b; Ray *et al.*, 1993)it is imperative that statistical analysis of results accommodates the possibility of phenotypic heterogeneity among the parents of children with clefts. Simultaneous analysis of maternal/paternal effects is also possible. Having failed to segregate the 83 parents in the present study into "natural" groupings of phenotypically similar individuals using the cluster analysis, an alternative multivariate statistical technique was applied, the Mahalanobis distance analysis. This is a measure of the degree of deviation of an individual's craniofacial morphology compared to the mean of the control population, and unlike the cluster analysis it enables degree of difference from the controls to be expressed numerically.

Differences in craniofacial morphology between the parental and control groups determined by two sample t-tests are located in all areas of the face; mandible, maxilla, cranial base and cranium, with different parameters being significantly different in the fathers and mothers. As such it was considered important to analyse each gender

separately. This study also differs from previous studies in that particular attention is afforded to the analysis of lateral cephalometric cranial as well as facial, morphology.

Fathers as a group showed a reduction in mandibular and maxillary area and reduction in palatal length. Mandibular differences have not been highlighted as being significantly different in previous parental studies, except for that by Prochazkova and Tolarova (1986) who noticed a reduced mandibular body length (Go-Me) compared to their control while Nakasima and Ichinose (1983) reported a longer mandibular ramus length (Ar-Go) in their parental sample.

Maxillary differences receive much greater attention in the previous literature with the general picture of absolute or relative maxillary retrusion in the antero-posterior dimension. Fraser and Pashayan (1970), Coccaro *et al.* (1972), Kurisu *et al.* (1974), Shibasaki and Ohtsuka (1978) and Ward (1989), all reported either a reduction in the ANB angle, or reduced facial convexity or both in mixed cleft/mixed sex parental samples.. However only the first authors reported a significantly reduced SNA angle. Both SNA and SNB were slightly smaller, but only in the fathers of children with clefts in the present study, and not to a significant level. Prochazkova and Tolarova (1986), on the other hand found a significantly increased ANB angle and a more convex profile suggesting maxillary prominence in their sample of 40 Czechoslovakian parents compared to a control.

The reduction in maxillary area and palatal length (ANS-PNS) in fathers of cleft children is consistent with the findings of Coccaro *et al.* (1972) and Nakasima and Ichinose (1983) for their overall parental samples and this is presumably a contributing factor to the reduced convexity of the facial profile which they reported. However Prochazkova and Tolarova (1986) found that their sample had longer palates (ANS-PNS) and a recent study by Raghavan *et al.* (1994) of thirty eight sets of parents with CL(P) children from India, also reported an increase in the palatal length.

Cranial base angle of the paternal group in the present study was reduced relative to the control (but this was significant only at the 5% level, $p = 0.036$).

Coccaro *et al.* (1972) also found a reduction in the cranial base angle of the parents overall, but Shibasaki and Ohtsuka (1978), Nakasima and Ichinose (1983) and Raghavan *et al.* (1994) all found it to be more obtuse in their combined parental samples.

Although there are no comparative figures from previous studies for cranial area there was a highly significant reduction in paternal cranial area compared to the control, which Nakasima and Ichinose (1984) also reported using a different method of analysis. They also reported a reduced cranial length in the antero-posterior dimension as did Blanco *et al.* (1992). Nakasima and Ichinose (1984) also reported that although the parental skull tends to be smaller in the vertical and the antero-posterior dimension, width parameters such as inter-orbital width, inter-zygomatic width, bigonial width and nasal cavity width, tend to be greater. Unfortunately in the absence of poster-anterior cephalograms, it was not possible to measure these parameters in the present study.

Analysed separately the maternal sample also showed significant differences compared to the female control group. Total anterior face height (N-Me) was greater in mothers of cleft children in keeping with the findings of Fraser and Pashayan (1970), Prochazkova and Tolarova (1986) and Sato (1989). There was no difference in upper face height (N-ANS) and a smaller UFH:LFH ratio implied that lower face height (LFH) was disproportionately increased. Prochazkova and Tolarova (1986), Sato (1989) and Ward *et al.* (1989) all reported an increase in lower face height. Interestingly, as in the present study Prochazkova and Tolarova (1986) found this increase in females, i.e. mothers only. Contrasting findings regarding face height can also be found in the literature. Studies by Kurisu *et al.* (1974), Blanco *et al.* (1992) and Raghavan *et al.* (1994) all reported a reduction in total face height, while Nakasima and Ichinose (1983) reported a reduced lower face height in their parental samples.

One variable about which there is no contradiction from any of the studies carried out to date is the upper face height, which is reported to be consistently shorter in the parents. This is in keeping with the congenital maxillary hypoplasia theory which is though to contribute to the concave profile in cleft lip and palate probands (Ross and Coupe, 1965; Bishara, 1979).

Regarding the mandible there was a significant increase in the length of the maternal mandibular ramus (Cd-14), which was in agreement with the finding of Nakasima and Ichinose (1984) who found an increase in a similar ramus length measurement (Ar-Go) in their overall parental sample. This tends to mitigate the effect of the larger lower face height on the maxillary mandibular planes angle which remained normal. The total mandibular length (Cd-Gn) was also significantly increased relative to the controls, an observation confined to the mothers in this study, and not noted in previous studies.

The size of the cranial base also differed in the mothers compared to the controls in that both the anterior cranial base length (S-N) and clivus length (S-Ba) were increased, but the saddle angle (N-S-Ba) remained the same. Prochazkova and Tolarova (1986) also reported an increase in the anterior cranial base length, while many other studies comment on the relative stability of cranial base parameters.

Finally, as in the paternal sample there is a highly significant reduction in maternal cranial area (c6). Also the frontal subtenuce measurement on the lateral cephalograms is reduced while the occipital subtenuce is longer. Figure 9 illustrates these differences which are due to differences in cranial outline shape. Subjectively there is a flatter profile of the frontal bone, while the occipital convexity is greater. The difference in these cranial measurements reached a high level of statistical significance, and parental craniofacial size and morphology merit further investigation; in respect of their role as genetic markers or predisposing factors in clefting.

Mahalanobis distances were used to determine whether there are differences between the parental and control groups and between maternal and paternal groups within the parents. This revealed a highly significant difference between parental and control craniofacial morphology, but also that the maternal group in the parental sample showed a significantly greater deviation from the female control than did the paternal group from the male control (see Figures 33(a) and (b)).

In addition this study has shown that the parameters which differentiate best between CP and CL(P) are mandibular length (Cd-Gn), ramus length (Cd-14), mandibular area and cranial area (c6), these being greater for CP. Furthermore the

stepwise discriminant analysis showed that the one single parameter which discriminated best was the mandibular ramus length (Cd-14), being an accurate predictor in 71.4% of CP and in 62.5% of CL(P) cases.

Among the parameters which Ward *et al.* (1989) found to be reduced in the CL(P) group were mandibular ramus length and total mandibular length when compared to a control. This begs the question regarding craniofacial morphology in the two cleft groups .... can mandibular dimensions be the most reliable distinguishing factor between CP and CL(P)?

It is interesting to note that the Mahalanobis distance was greater for females than for males and one of the most significantly different parameters when comparing parental and control data ($p = 0.011$) is the disproportionate increase in the maternal mandibular length (Cd-Gn). The maternal parameters which differed in the study by Figalova *et al.* (1974) on a combined sample of parents of CP, CL and CLP probands were also cranial and mandibular; and Blanco *et al.* (1992) found that only the female relatives of probands with cleft lip and/or palate had significant reductions in certain cephalometric craniofacial parameters such as cranial height, cranial width and anterior face height. Other parameters which were significantly different in female parents in the present study were a reduction in cross sectional area of the cranium as measured on lateral cephalograms, a longer anterior cranial base (S-N), a longer clivus length (S-Ba) and an increased anterior face height (N-Me). In view of the well documented female predilection to isolated cleft palate, this could prove to be a significant observation when considering genetic predisposition. Perhaps the craniofacial morphology conferred via the maternal genome renders a female embryo, in which shelf elevation is delayed relative to the male more susceptible to a disruption in that process.

The present study is uniquely relevant to comments in the literature regarding the nature of the craniofacial morphological differences between cleft probands and controls. There is occasional speculation about the relative contributions of heredity and environment in the production of the different cleft phenotypes which have been well characterised over the years by numerous workers, a number of whom, Dahl (1970), Bishara (1973), Bishara *et al.* (1976, 1979, 1985), Smahel (1984a, b, c) and Semb

(1991a, b) have published particularly thorough studies on this subject. Clues regarding the relative contributions of heredity and environmental factors in clefting may emerge from the study of parental craniofacial morphology and may also shed some light on the aetiology and pathogenesis of CP and CL(P).

The qualitative data on the deviation in craniofacial morphology noted in probands with clefting tends to be more consistent than that for their parents (as discussed below and summarised in Table 27). Indeed it was the discovery of the characteristic craniofacial phenotype in cleft probands that inspired the study of parental craniofacial form, the objective being to elucidate whether or not it was due to heredity factors (Fraser and Pashayan, 1970). An alternative hypothesis is that there is an overpowering environmental influence on morphology post-natally due to the presence of the cleft defect and the resulting functional abnormalities, along the lines of the functional and capsular matrices as proposed by Moss (1968, 1969). Such influences as the altered oronasopharyngeal function or the periosteal matrices which affect bone resorption and deposition and therefore size and shape may result in morphological changes. Surgical repair of the cleft is a further complicating factor and where deviations in craniofacial morphology have been recorded they have often been considered secondary to the repair of the cleft in the growing child. (Mestre *et al.*, 1960; Harvold 1954, 1960, 1961; Lambadusuriya *et al.*,, 1988; Mars and Houston, 1990; Semb, 1991).

This was not, however, borne out by the Dahl study (1970) which involved a comparison of operated and unoperated clefts in the CP and CL(P) categories (Bishara, 1973). Dahl concluded that surgical closure of the palate had little influence on the basal structures of the face apart from maxillary width in the CL(P) cases and that sequelae of palate repair were practically restricted to the palate and dento-alveolar areas both in subjects with isolated CP and in subjects with CL(P). Bishara (1973) also studied iatrogenic effects on a sample of 20 Caucasian females with isolated CP and found that both operated and non-operated groups of CP patients had bimaxillary retrognathia and concluded that this was a morphogenetic tendency.

Table 27. Cephalometric investigations into the craniofacial morphology of probands with orofacial clefting.

	CL	CLP			CP		
Author	Dahl	Dahl	Smahel	Semb	Dahl	Smahel	Cronin and Hunter
Year	1970	1970	1986	1991	1970	1984	1980
Race	Danish	Danish	Czech	Norwegian	Danish	Czech	Caucasian
Number	n=62	n=153	n=30	n=257	n=57	n=90	n=38
Age/Sex	Adult males	Adult males	5 year old males	25 year study, both sexes	Adult males	Adult males	19 pairs of twins, both sexes
Type	CS/OP	CS/OP	CS/UO	L/OP	CS/OP	CS/OP	CS/OP
Mandible							
LFH	o	+	o	+	+	+	+
Gonial ∠	+	+	+	+	+	+	+
Ramus	-	-	-		-	-	-
Body	-	-	-		-	-	-
SNB	o	-	-	-	-	-	-
Maxilla							
UFH	-	-	-	-	o	o	
ANS-PNS	-	-	o	-	-	-	-
PFH	-	-	-		-	-	-
SNA	o	-	-	-	-	o	-
Facial							
TFH	-	+	o	+	+	o	+
ANB	o	-	o	-	-	o	-
MMPA	+	+	o	+	+	+	+
C Base							
S-N		-	-	+	-	-	-
S-Ba	-	-	-		-	-	o
N-S-Ba	o	+	o	+	+	-	+
Cranium							
Height	-	-	-		-	-	
Length	-	o	o		-	o	
Fr. chord	-	-			o		
Occ. chord	-	-			-		
Par. chord	-	-			-		

KEY: CS = cross-sectional OP = operated + = increased

L = longitudinal UO = unoperated - = reduced

o = no different

Analysis of the evidence for each of these viewpoints requires a multifactorial analysis of the available literature. Many of the relevant studies combined all clefts and both sexes for their statistical analysis.

The remainder of this discussion will be concerned with the craniofacial form of the basal craniofacial structures in cleft patients for the purpose of comparison with the parental values in the present study. These basal structures are thought to have a morphogenetic component. The studies referred to involve patients who have been subjected to surgery in the Dahl (1970), Smahel and Brejcha (1983, 1984 a, b, c, 1985) and Semb (1991a) studies, while the Bishara (1979) and Mars (1992) studies referred to occasionally are concerned with unoperated clefts. Other studies are quoted at the relevant points in the discussion.

The most striking feature of previous studies on the cephalometric features of patients with clefting deformity is, with the very occasional exception, the relative consistency of many of the findings (see Table 14). In addition there are many similarities between the general skeletal features of those affected by CP and by CL(P) and there is general support for the concept that disturbance in the morphology of the mandible and middle third of the face varies with the severity of the cleft being least for cleft lip (CL) and greatest for bilateral cleft lip and palate (BCLP) (Cronin and Hunter, 1980; Smahel, 1984c). This is well illustrated by superimposition of the mean facial diagrams for each category of cleft produced by Dahl (1970), or the cleft/control superimpositions in the Ross and Coupe (1965) and Cronin and Hunter (1980) twin-pair studies.

A reduction in horizontal and vertical dimensions of the middle third of the face, and/or a flatter profile, is often a clinically apparent feature of the cleft child or adult. In this respect a reduced palatal length and reduced posterior face height are very consistent findings in all categories of clefting (Moss 1956; Ross 1965; Dahl 1970; Bishara, *et al.*, 1979). Anterior upper face height is also reduced in all except the isolated CP category (Dahl, 1970; Smahel, 1984a; Semb, 1991a; Trotman *et al.*, 1993) and posterior displacement of the maxilla (i.e. reduced SNA angle) is seen in UCLP and BCLP, but not in CL or isolated CP (Dahl, 1970).

Smahel and Mullerova (1986) noted the vertical midface reduction in CLP patients prior to palatal repair suggesting an impairment in the interaction between the maxilla and the growth regulating nasal septum or a primary hypoplasia or tissue deficiency not related to palate surgery. This view was also expressed by Bishara (1973) in a comparison of operated and non-operated CP and CLP subjects, both of which had midface retrusion; while Ross and Coupe (1965) in a study of twins discordant for CL(P) concluded that the underdeveloped maxilla is due to an inherent developmental fault, which leads to an intrinsic growth deficiency (Ross, 1991). The latter view of morphogenetic maxillary growth deficiency receives a degree of support from the present study which demonstrates a reduced palatal length (ANS-PNS) and a tendency to reduction in SNA in the non-cleft fathers of children with clefting deformity.

An increased anterior total face height is another consistent feature in all clefts apart from cleft lip (Dahl, 1970). This is invariably due to an increase in the lower face height with the upper face height being reduced (in CP, UCLP and BCLP) or normal (in CP). The mothers of cleft children in the present study showed similar characteristics suggesting the possibility of a hereditary component in this feature.

Deficient growth of the mandibular ramus and body (Borden, 1957; Levin, 1963; Dahl, 1970; Bishara, *et al.*, 1979 and Smahel, 1984 a,b,c,) and obtuse gonial angle is consistently observed in all reported studies in the literature to varying extents in all cleft groups (and even in the UCLP patients prior to palatal repair, Smahel and Mullerova, 1986). Studies looking at isolated CP have speculated that this is due to some form of environmental or genetic teratogenic agent. Further evidence of the role of a small mandible in CP is provided by experiments on laboratory animals (Jelinek and Peterka, 1977; Diewert, 1979). Furthermore Smahel (1984a) discovered that in general the less extensive the degree of palatal clefting the greater the mandibular deficiency. This was explained by Jelinek and Dostal (1983) as being due to a more potent teratogenic agent. Induction of a soft palate cleft is chronologically later when palatogenesis is well advanced and malformation could only be induced by a strong teratogenic impetus at that stage.

Previous studies on parental mandibular morphology however, fail to show similar features. In the few instances where mandibular differences were noted, only that by Prochazkova and Tolarova (1986) showed a more obtuse gonial angle, and Nakasima and Ichinose (1983) reported a shorter body length (Go-Me) but an increased ramus length (Ar-Go). Neither the fathers nor mothers of cleft children in the present study showed a reduction in mandibular dimensions, and in fact the CP mothers showed a significant increase in mandibular ramus length (Cd-14) and in total mandibular length (Cd-Gn). The absence of any evidence that mandibular morphology in clefting is of a hereditary nature suggests that there may be some form of teratogen in action. It could be that a teratogenic insult at the critical stage of fusion of the primary or secondary palate simultaneously disturbs growth of the developing mandibular anlage, resulting in the characteristically reduced dimensions in CP and CL(P) probands. A weaker teratogenic action may be responsible for CL alone such that no disturbance of mandibular growth occurs, which would explain the normal mandibular morphology in cleft lip (Dahl, 1970; Smahel, 1984c).

Differences in cranial parameters in both parents and probands compared to controls were also found to exist in the few studies that incorporated cranial measurements in their analysis. Dahl (1970) measured head circumference and head length (from glabella to opistho-cranion) as anthropological measurements in all cleft groups and found both parameters to be invariably reduced. He also measured the frontal (N-Br) parietal (Br-L) and occipital (L-Ba) cranial cords and with the exception of the frontal chord in isolated CP he found significant reductions in all cases. Smahel (1984a) also found a reduction in the height of the neurocranium (Br-Ba) in isolated CP cases.

A similar trend in the reduction of cranial parameters was noted by Nakasima and Ichinose (1984) who measured cranial area and cranial length; and the present study found significantly reduced cranial area in both fathers and mothers compared to their respective controls. Frontal chord and subtenuce measurements also tended to be smaller, in the parents but interestingly in both parental groups the occipital subtenuce measurement was significantly larger. This supports a definite difference in cranial morphology in the occipital region with a tendency to occipital bossing or a prominent

external occipital protuberance. It is quite feasible that the cleft phenotype includes a reduced occipital chord measurement (as Dahl (1970) noted in cleft patients) and simultaneously an increase in the occipital subtenuce distance.

The majority of the studies which examined the craniofacial morphology of subjects with clefting included cranial base parameters; but no overall consensus on which aspects, if any, differ from a control has been reached. In fact many thorough studies conclude that there is no difference whatsoever between the cranial base in controls and cleft patients, e.g., Bjork (1961), Brader (1957), Engman (1965) and Smahel and Mullerova (1986).

Dahl (1970) found that all cleft categories had a shorter clivus length (S-Ba) and that CP and CLP patients (but not CL) also had a reduced anterior cranial base measurement (S-N). The studies on CP and CL(P) patients carried out by Smahel in 1984 and 1986 respectively were in agreement as regards the anterior and posterior cranial base shortening. Other studies such as those by Sandham and Cheng (1988) on CL(P) patients found the clivus length (S-Ba) reduced but not the anterior cranial base length (S-N), while Trotman *et al.* (1993) in a CL(P) monozygotic twin pair study found the converse to be true i.e. the S-N was smaller but the S-Ba measurement was normal. Krogman's study of 102 cleft children (59 CP, 43 UCLP) from birth to six years found cranial base parameters were increased relative to a control (Krogman, 1975). This was supported by the findings of Semb (1991a) who reported an increase in anterior cranial base length (S-N) in a 25 year study of 257 UCLP patients in Norway.

The present parental study, however, found that sex differences were apparent in cranial base size since the anterior cranial base (N-S) and clivus length (S-Ba) were increased in the mothers of children with cleft lip and/or palate, while there was no difference in the fathers compared to the male control group. Only one other parental study, that by Nakasima and Ichinose (1983), reported a significant difference in cranial base in their sample of 502 Japanese parents (450 CL(P) and 52 CP). They were found on average to have a longer anterior cranial base measurement (S-N), but this study did not carry out a separate analysis on fathers and mothers or CP and CL(P) separately.

The overall picture from previous work is that the cranial base size is fairly stable, and in the studies which did report a deviation in length there is no consistent agreement.

Finally, studies on craniofacial morphology often include analysis of cranial base angulation because of its implication in the relationship between maxillary and mandibular skeletal bases (Kerr and Hirst, 1987). Smahel (1984 b, c) noted a more acute cranial base angle in CP patients and no change in the CL(P) group. Krogman *et al.* (1975) and Bishara *et al.* (1979) on the other hand found that the cranial base angle was more acute in samples of CL(P) children and adults respectively. These studies supported the conclusion from an earlier study by Moss (1956) on a sample of 103 isolated CP cases that "dysostosis sphenoidale" resulting in acute cranial base angulation is a feature of clefting.

The lack of consensus in the literature could well be due to the fact that there is a real sex and/or cleft type effect in the morphology of the cranial base, especially cranial base angulation; and due to the pooling of data in many of the studies this effect is masked. Ross (1965) found a sex difference in cranial base angulation with the female having a larger N-S-Ba measurement and also that CL(P) patients tended to be larger than CP for this parameter. Sandham and Cheng (1988) also separated their mixed sex sample of CL(P) patients and found that only the females had a significantly more obtuse cranial base angle, the males being normal in this respect; while Smahel (1984 b, c) looked at CP and CL(P) separately and was in agreement with Ross (1965) that there was a more acute cranial base angulation in isolated CP, but not in CL(P).

If, indeed there is a gender/cleft type difference in cranial base angulation with females and CL(P) being larger it could be that random CL(P) samples tending, as they do, to have more males will mask this effect. The Semb (1991a) study of 257 UCLP subjects, 176 of whom were male, were reported to have a more obtuse cranial base angle overall which would not be incompatible with this hypothesis. Conversely females and CP would tend to cancel out the effects of one another in samples where they were not separately analysed.

In the context of the present study it is interesting to note that a gender difference was detected with the cranial base angulation in that the cranial base angle (N-S-Ba)

was more obtuse in the mothers than in the paternal sample; and in comparison to the controls the fathers had a more acute cranial base angle than the male control, while no difference was detected between the mothers and female control subjects. Coccaro *et al.* (1972) examined 40 Caucasian parents (20 fathers, 20 mothers) of CL(P) children and found them to have a more acute cranial base angle, while the Nakasima and Ichinose (1983) study in Japan on 502 parents, some of whom had isolated cleft palate children reported an increase in the cranial base angulation.

There is some evidence from this study that mothers of cleft children and CL(P) probands may have more obtuse cranial base angulation compared to fathers and isolated CP probands respectively. However, there is insufficient evidence available from parental and proband studies which differentiated between cleft type and gender to draw firm conclusions. The cranial base is believed to be under genetic control, but variation in morphology in craniofacial deformities could also be explained by teratogenic action.

The cumulative evidence from many previous studies leaves little doubt that differences do exist in both parental and proband craniofacial morphology, and that the latter is not entirely due to postnatal environmental or iatrogenic effects. The question which arises in respect of the aetiopathogenesis of clefting is whether there is evidence for genetic determination of the typical cleft craniofacial morphology or whether this is more likely to be due to some teratogenic influence acting at some critical stage in intra-uterine embryonic development.

The overall conclusions from this comparative analysis of cleft parents and probands is that there are similar trends in craniofacial morphology in maxillary and cranial parameters, suggesting that the characteristic cleft, cranial and maxillary morphology in cleft probands may be inherited. However, the evidence from previous cephalometric studies including the present study suggests that the mandibular morphology in clefting is not inherited. The present study also indicates the possibility of differences in morphology according to cleft type, or perhaps gender (e.g., in the cranial base angulation), but there is insufficient evidence available to draw firm conclusions.

The deviations in proband craniofacial shape could therefore represent either or both of the following;

(a) the result of teratogenic action acting simultaneously to produce the cleft defect and a disruption of development of other craniofacial structures,

(b) the genetically conferred craniofacial characteristics passed on from the parents, which may represent the extreme limit of normal variability and exceeding the liability threshold for normal primary or secondary palatogenesis.

In contemplating the aetiology of clefting the evidence from previous cephalometric studies and a knowledge of the embryology of the affected structures enables a good insight into this subject.

The maxilla and mandible form from the first branchial arch, becoming discrete processes during the fourth week of embryonic life. The paired mandibular processes merge with each other in the fourth week and give rise to the lower jaw and lower lip. The maxillary processes form the secondary palate (which fuses during the ninth to twelfth week), the upper jaw and the lateral portions of the upper lip. The cranial base forms by the fusion of several cartilages to form the sphenoid, temporal and occipital bones. Fusion begins during the sixth to twelfth week, and seems to be affected during the latter half of this period during fusion of the secondary palate and growth of the jaws and cranial base. Furthermore Diewert (1985) has shown that rapid embryonic growth of the craniofacial structures occurs between the sixth and twelfth week post-conception.

The discovery of a significant reduction in cranial size in both parents and probands is an interesting finding. The significance of cranial size and shape in the aetiology of clefting remains unclear. Reduction in cranial size in the cleft probands can obviously be explained by heredity since reduction in parental cranial size (in both fathers and mothers) was noted.

It seems reasonable in light of the temporal proximity of development of the various craniofacial parameters to consider the possibility of environmental effects as well as genetics for this association.

The detection of occipital and other cranial differences and the reported association of cervical vertebral anomalies with clefting (Sandham, 1986) suggests the possibility of environmental factors in the aetiology. This would provide further evidence for the teratogenic "field theory" of clefting whereby the effects of a teratogen acting at a critical stage in embryonic development and contributing to the aetiology of the cleft affects simultaneously developing craniofacial structures (Cohen, 1982; Kirby and Bockman, 1984; Mossey and Sandham, 1989).

At least three possible aetiologies for CP and CL(P) can be considered:

I) A single genetic malformation "syndrome" characterised by several noncontiguous malformation sequences in the same patient.

II) An environmental agent or agents, probably one which affects growth during the late embryonic period. It is feasible that if subjected to teratogenic activity, the whole midface complex, cranial base and upper cervical vertebrae components are vulnerable to anomalous development.

III) A combination of inherited tendencies or susceptibilities and environmental effects.

The overall conclusions from parent/proband studies are as follows:

I) There is a characteristic genetically determined craniofacial morphology in respect of cranium, maxilla and possibly cranial base which predisposes to the clefting deformity.

II) Certain craniofacial features are characteristic of either CP or CL(P) (e.g., maxillary retrusion with CL(P) but not CP) and of either one or other of the parents (e.g. more acute cranial base angle with fathers but not mothers of cleft children).

III) Mandibular morphology presents an apparent paradox in orofacial clefting. Although mandibular parameters were an apparently good discriminator between CP and CL(P) and maternal mandibular length was greater than in the female control population, there is no evidence for mandibular morphology in cleft probands being a hereditary characteristic. It is possible that the teratogenic action

which precipitates the cleft is also responsible for the mandibular hypoplasia. This is in keeping with the "field theory" of orofacial clefting.

IV) Clefting is therefore likely to be the result of a combination of a genetically predisposed craniofacial morphology precipitated by an environmental teratogen.

V) Further studies designed to differentiate between CP and CL(P) by means of craniofacial parameters are required e.g., use of postero-anterior analysis to determine width differences.

VI) Study of craniofacial shape, rather than just size and area may yield further valuable information.

4.2 GENETIC RESULTS

The present study sought to provide further insight into the hereditary aspect of this association by examining the DNA of the parents of children with clefting for the prevalence of the various TGFα RFLPs. Parents of children with different types of cleft, CL(P) and CP were included in the study sample so that possible differences between the different cleft groups could be highlighted. The results are discussed in the light of previous studies which investigated association or linkage between TGFα and clefting (Table 28).

The finding of a significant association between CL(P) and the *Taq*I C2 allele of the TGFα gene is consistent with previous studies by Ardinger *et al.* (1989), Chenevix-Trench *et al.* (1991), Holder *et al.* (1992), Stoll *et al.* (1992) and Sassani *et al.* (1993). Ardinger *et al.* (1989) hypothesised that there might be a non-random association between clefting and RFLPs of candidate genes which have a role in palate formation. They reported a significant association between two RFLPs of transforming growth factor alpha (TGFα) and clefting in a sample of 80 patients with nonsyndromic CL(P) and 102 controls. ($p = 0.0047$ for the *Taq* C2 allele, and $p = 0.0052$ for the *Bam*HI A1 allele).

Only one previous study looked exclusively at an isolated CP sample (Shiang *et al.*, 1993) and the significant association between the *Taq*I C2 allele reported in their sample of 52 patients (86 chromosomes) was reproduced in the present parental study. The present study was the first to report a significant association between CP and the *Bam*HI A1 allele. Ardinger *et al.* (1989) did report an association between CL(P) probands and the same A1 allele, as did Stoll *et al.* (1992) in a mixed sample of CL(P) and CP probands. Shiang *et al.* (1993) found no association with the *Bam*HI polymorphisms on his smaller sample of CP probands (32 chromosomes).

Three aspects of the above results are worthy of further comment.

i) Familial v non-familial clefting.

ii) Parental v proband allele frequency.

iii) Dual aetiology.

Table 28 Previous studies on TGFα in clefting

Author (year) Location	Cleft Abnormality Sample Size	Familial (% of sample)	Association Alleles Genotype
Ardinger *et al.*, (1989) Iowa, USA	CL(P) n=80	40%	A1, C2
Chenevix-Trench *et al.*, (1991), Australia	CL(P) n=92	50%	C2
	CP n= 4		
Hecht *et al.*, (1991) Texas, USA	CL(P) 11 families	All	No evidence of linkage
Stoll *et al.*, (1992) France	CL(P) n=67	None	A2
	CP n=38		
Holder *et al.*, (1992) UK	CL(P) n=60	37%	C2
Vintiner *et al.*, (1992) UK	CL(P) 8 families	All	No evidence of linkage
Sassani *et al.*, (1993) Pennsylvania, USA	CL(P) n=100	15%	C2
Jara *et al.*, (1993) Chile	CL(P) n= 21	?	A1, A2 tested no association
Shiang *et al.*, (1993) Iowa, USA	CP n= 52	None	C2
Present Study (1994)	CL(P) n= 41	7·5%	C2
West of Scotland	CP n= 35	14%	A1, C2

I) Familial v non-familial clefting.

The implication of a major gene locus in CL(P) by complex segregation analysis, prompted some workers to look for an association between the TGFα RFLPs and family history. Ardinger *et al.* (1989) reported a significant difference in haplotype distribution when comparing those CL(P) probands with and without a family history ($\chi^2 = 6.42$, $p = 0.04$) with an over-representation of the C2 A2 B2 haplotype in cases reporting a family history. Holder *et al.* (1992), however, also reported an over-presentation of the C2 A2 B2 haplotype in the absence of positive family history. Subsequent linkage studies also failed to support this observation, and in fact they exclude TGFα as a major gene in these tested families. For example, Vintiner and Holder and their colleagues in London found an association between TGFα in a group of individuals with CL(P) (Vintiner *et al.*, (1992) but found no evidence of linkage when multiplex families were studied (Holder *et al.*, 1992). Hecht *et al.* (1991) in a study of 11 CL(P) families also excluded tight linkage.

These results suggest the possibility that TGFα plays an epistatic role in the development of clefting, but that it is not the major gene.

II) Parental v proband allele frequency

It is important to bear in mind that whilst previous investigations into the relationship have logically used DNA from the cleft probands, the present study was mainly concerned with parental DNA. A few probands in the present study did volunteer a blood sample and these were simultaneously analysed, but the numbers are so small that no significant conclusions can be drawn from them.

The most striking feature of the comparison of the parental/proband data is the degree of similarity of the *Taq*I C2 allele frequency in the CL(P) group. In CP parents, however, the *Taq*I C2 allele frequency is also significantly increased, a finding not previously reported in proband studies when CP and CL(P) probands were grouped together for analysis; but which was reported by Shiang *et al.* (1993) in his exclusive sample of 52 CP patients. ($\chi^2 = 5.95$, $p < 0.05$). The *Bam*HI allele would also appear to be implicated in both CL(P) and CP. Ardinger *et al.* (1989) reported a significant association between this A1 allele and his sample of 80 CL(P) probands, while Stoll *et al.*

(1992) observed that this allele was not only significantly more frequent in CL(P) compared to his control ($\chi^2 = 4.02$ $p < 0.05$), but there was a bias towards BCLP ($\chi^2 = 5.95$, $p < 0.05$). In this West of Scotland parental sample no such association was found with CL(P), but the CP parents had a greater frequency of this *Bam*HI A1 allele compared to the control.

If these parental/proband allele frequency observations are borne out by further study on larger samples and in different populations, they may give a further insight into the aetiology of orofacial clefting. It is possible that a double-hit mutation at the TGFα locus predisposes to clefting. In the case of CL(P) a C2 parental/C2 child double-hit mutation may be the most potent predisposition, while an A1 parental/C2 child mutation may likewise predispose to isolated CP.

The TGFα allele and genotype frequency results in the present study could not contradict this theory with a significant increase in the frequency of the C2 allele in both parents and probands in CL(P); and a significant increase in the frequency of the A1 allele and the A1 A2 genotype among CP parents. In addition the A1 A2 genotype was under-represented in both the CL(P) proband and parent groups while there was a highly significant difference in A1 A2 genotype frequency between the CP and CL(P) groups ($\chi^2 = 7.29$ $p = 0.003$) It is also of interest to note that the A1 A2 genotype is predominant in the material genome in both cleft categories ($\chi^2 = 3.85$ $p = 0.049$) and A1 A2 was unrepresented in any of the 18 CL(P) fathers in the present study.

III) Dual aetiology.

There is an apparent dual genetic association between the C2 and A1 alleles and both types of orofacial clefting, neither polymorphism being exclusively associated with CL(P) or CP. Genetic and developmental differences however suggest a difference in the aetio-pathogenic mechanism, the difference in sex distribution with CL(P) more common in males and CP in females being a simple example. It is likely, however, that some common signals may affect formation of both the primary and secondary palate even though they are embryologically distinct. Van der Woude syndrome is an example where an autosomal dominant disorder of lower lip pits and orofacial clefting can manifest either as CP or CL(P) within the same family (Burdick *et al.*, 1985). This

indicates that the same mutation in a single gene can affect primary and/or secondary palate formation.

Association studies including the present study, investigating the role of TGFα in clefting have provided evidence that TGFα or a gene in the region of the TGFα locus are involved in a common genetic mechanism influencing both primary and secondary-palatogenesis. TGFα was selected by Ardinger *et al.* (1989) as a candidate gene in the initial association study on CL(P) because of its expression in palatal tissue in culture. (Ferguson, 1987; Dixon *et al.*, 1991) subsequently demonstrated that TGFα was present at high levels in the MEE of the palatal shelves at the time of secondary palate fusion. Shiang *et al.* (1991) used a series of twenty overlapping PCR primers for TGFα with DNA to carry out a search for more highly associated DNA polymorphisms using single strand conformation polymorphism (SSCP) analysis. They studied a group of 115 CL(P) and 25 CP patients and that a three-allele SSCP showed a significant association with CL(P) ($p = 0.04$) and an even more significant association with CP ($p = 0.001$). This suggests that the causal mutation in the TGFα gene is in or near the proximal 3' region overlapped by the PCR primers. This is further evidence of genetic homogeneity for CO and CL(P) at least at the TGFα locus.

Another means of reinforcing the evidence implicating a particular gene is by means of linkage analysis whereby pedigrees are scrutinised for evidence of concordance or discordance of inheritance from an affected parent of the haplotypes of interest. While the demonstration of association between TGFα RFLPs as defined by digestion with *Bam*HI and *Taq*I in seven independent studies with two cleft phenotypes is strongly suggestive of its role in two independent attempts to confirm the association studies by using linkage analysis have failed (Hecht *et al.*, 1991; Vintiner *et al.*, 1992), (see Table 28).

Both of these studies assumed, for the purpose of linkage analysis, that CL(P) was due to an autosomal dominant gene with a high degree of penetrance (chosen with reference to an accompanying segregation analysis in the Hecht study). Effectively they were testing whether all the genetic variance in their multiplex families can be explained by the effects of TGFα as the single susceptibility locus. In addition they were both

carried out on a small number of families. Failure to detect linkage could therefore be explained by the likely heterogeneity of nonsyndromic CL(P) and the relatively small samples lacking the statistical power to confirm linkage (Farrall *et al.*, 1993; Hecht *et al.*, 1993). Such results, although valuable as indicators for future research, should not be accepted as definitive pronouncements. The exact nature of the role of TGFα in the light of the aforementioned association and linkage studies still therefore remains obscure. Additional multiplex CL(P) and CP families with C2 and A1 alleles are required to confirm or refute the evidence that presently exists to implicate the TGFα gene in orofacial clefting. Ideally this should involve families which include a substantial number of affected individuals which improves the chances of inclusion of "monogenic" cases, and reduces concerns over aetiological heterogeneity. Such families would have sufficient power to accept or reject linkage by themselves.

It is important to note in this respect that from population prevalence studies and sib recurrence risk the TGFα susceptibility locus determines only a minor fraction of the familial recurrence of CL(P), between 2.5% and 5.6% (Farrall *et al.*, 1993). This point has also been noted by Mitchell and Risch (1992) and new polymorphisms may be found that show a stronger association with CL(P). For example a linkage study by Eiberg *et al.* (1987) in Denmark on a combined CP/CL(P) sample of 58 pedigrees (carefully selected for a distribution suggestive of autosomal dominance) indicated close linkage with the blood clotting factor XIIIA (F13A). Also a three-allele single strand conformation polymorphism reported by Shiang *et al.* (1991) shows a stronger association.

In an overview of mechanisms in embryogenesis, Ferguson (1994) considers the evidence provided by transgenic gene knock-out experiments (see section 1.2.5.1) in which disruption of supposedly important genes often produces a minimal phenotype. He provides several possible explanations. One of these which may have implications for the TGFα gene and clefting is the concept of "maternal rescue"; whereby maternal proteins can cross the yolk sac and placenta to the developing embryo. The TGFβ1 knock-out mice produced by Shull *et al.* (1992) and Kulkarni *et al.* (1993) which survived for three weeks did so only because of maternal milk. It is also known that

TGFα which is implicated in palatal clefting can cross from mother to embryo; and Ferguson (1994) hypothesises that for cleft palate to occur a two-hit mutation may be necessary mutation of the maternal growth factor and of the embryonic growth factor. It is known that polygenic multifactorial birth defects such as CP and CL(P) tend to associate more closely with the mother, the traditional explanation being that they are exposed to maternal metabolic/environmental factors during pregnancy. This is certainly the case, but the presence of two mutations, one in the maternal and one in the embryonic genome would provide an alternative explanation.

The data from the present study does not contain sufficient family pedigrees to support or refute this hypothesis with respect to TGFα. All that can be said is that of the 19 pedigrees where the child had agreed to provide a DNA sample for analysis, their was no evidence of a consistent double hit maternal/embryo mutation at the C2 allele locus. Of the six pedigrees where the cleft proband had the C2 allele, only three of these showed that the maternal genome had contributed the C2 allele to the proband. In a further three cases the proband C2 allele was apparently inherited from the father.

A double hit maternal/embryo mutation at the TGFα A1 locus could not be ruled out from the data available, but this could only account for three of the 18 probands whose A1 allele status could be ascertained. The remaining 15 probands did not demonstrate the A1 allele. No instance of father to proband A1 allele transmission was noted among the present families.

The TGFα association studies which did report a significant association between the C2 and A1 alleles and clefting could account for only a portion and not all cases of clefting. None of these reports provide parent/proband data to differentiate between maternal/paternal hereditary origins of the TGFα polymorphisms. Larger studies on the human population with more complete pedigrees and other candidate genes will be required to further knowledge in this intriguing area of cleft lip and palate research.

4.3 CROSS CORRELATION OF PHENOTYPE/GENOTYPE DATA

Analysis of cephalometric craniofacial parameters in this study has confirmed that distinctive differences do exist between the parents of children with clefting deformities and a control. Furthermore there are differences (a) between the parents of CP children and those of CL(P) children and (b) between mothers and fathers with regard to craniofacial morphology. Having also ascertained definitive differences in allele frequency and genotype at the TGFα locus compared to a control in these same parents, and genetic differences according to cleft type it seems logical to investigate whether the parental genotype bears any relation to their craniofacial form or phenotype.

The results of this parental craniofacial morphology/parental genotype study comprehensively confirms the null hypothesis that there is no difference between the craniofacial morphology conferred by different genotypes at the TGFα locus. There is undoubtedly an association between parental craniofacial morphology (phenotype) and predisposition to clefting and an association between parental TGFα allele frequency and genotype and predisposition to clefting but no phenotype/genotype association.

Incidentally many of these plots simultaneously demonstrate CP and CL(P) parent differences which were expected findings in view of the differences highlighted in the CP/CL(P) discrimination using craniofacial measurements. This genotype/phenotype comparison was primarily concerned with the detection of craniofacial morphological features conferred by a particular genotype and therefore craniofacial differences due to cleft type which were analysed in section 3.1.1 are not commented upon here. It is possible that a genotype/phenotype interaction may operate for the predisposition to either CP or CL(P). For example if a father possesses the C1C1 genotype, then the size of a particular craniofacial parameter (e.g. cranial height) may discriminate between the likelihood of producing a CP or CL(P) child. The number of subjects involved in the present study when subdivided for cleft type, sex and genotype precludes a meaningful analysis of this possibility.

These results suggest that there are certain morphogenes involved in determining the cleft susceptible parental craniofacial morphology, but there is no evidence that these

are the same genes as those at the TGFα locus which have been found to be significantly associated with CP and CL(P).

Despite the fact that no association between TGFα genotype and craniofacial morphology was found in the parents of children with clefting, the TGFα genotype can nevertheless be used (a) to determine the likelihood of an individual in the population having a child with a cleft defect and furthermore (b) to discriminate between the likelihood of that birth defect being a CP or CL(P).

In order to obtain the information required for this genotypic determination of liability to clefting, characterisation of the TGFα locus by restriction enzyme digestion using all three enzymes is required. Firstly *Taq*I RFLPs will determine whether an individual is predisposed towards having a child with clefting; and *Bam*HI/*Rsa*I interaction can subsequently be used to determine whether that predisposition is towards a child with CP or with CL(P).

The discrimination between the two types of birth defect can be further sharpened by incorporating cephalometric data, and four craniofacial parameters have been identified by this study as being particularly useful for this purpose. Being able to reduce the number of craniofacial parameters to four represents a considerable simplification of the cephalometric analysis procedure normally required for analysis of craniofacial form. In this respect it may be somewhat surprising that some of those variables found earlier to be important in discrimination do not feature here. This may be explained by the fact that their contribution to the discrimination is accounted for by their correlation with genotype.

4.4 PRACTICAL IMPLICATION OF THE PRESENT STUDY.

Despite advances in surgical and psychological management, considerable confusion remains about the genetic implication of clefting, and about the distinction between CP and CL(P) in terms of aetiology and pathogenesis. Contemporary expert opinion on the aetiology of clefting is that CP and CL(P) are anomalies with a genetic predisposition and a contributory environmental component. The relative contribution of each factor in a particular case are usually vague and so genetic counselling for "at risk" individuals is equally vague based on average recurrence risks. Some families may go on to have further affected children, thus revealing themselves to have a significant genetic predisposition. Certain genes have been identified, by their known function in palatogenesis in either humans or animals, as being "candidate genes" for clefting among which is TGFα.

The present study has taken the unprecedented step of looking for and identifying genetic markers at the TGFα locus in parents of children with CL(P) and CP. This helps to distinguish the high risk from the low risk couples before an affected child is born. In addition there are certain phenotypic markers in the craniofacial morphology which characterise these higher risk parents. Furthermore there are both phenotypic and genotypic markers which discriminate according to cleft type (CP or CL(P)).

The practical implication of being able to identify these TGFα polymorphisms, is that the accuracy of genetic counselling in orofacial clefting is improved, and present knowledge of the aetiopathogenesis of clefting is enhanced. The lack of correlation between the TGFα genotypes and the significantly different cephalometric parameters would suggest, in line with current thought, that other genetic loci are involved in the predispostion to CL(P) and CP.

The associations reported here identify the West of Scotland population as being genetically at risk for CP and CL(P). Further investigation using the methodology described here and other candidate genes would merit consideration.

APPENDIX A

SUBJECTS IN PARENTAL SAMPLE AND CONTROL GROUP

I.	Subjects involved in cephalometric study:	Parents
		Control
II.	Subjects involved in genetic study:	Parents
		Control

A.1 PARTICIPANTS IN THE LATERAL CEPHALOMETRIC STUDY: PARENTS.

Record		Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
I	21	CP	1	2	1	560411	920401	36.0	10.115
I	22	CP	2	1	1	580429	920401	33.9	10.115
I	23	CL	3	2	1	480306	920401	44.1	10.115
	24	CL	4	1	2	461014	920401	45.5	9.020
	25	CLP	5	2	2	590927	920410	32.5	9.260
I	26	CLP	6	1		590927	920410	32.5	10.115
I	27	CLP	7	2	1	531213	920415	38.3	10.115
	28	CLP	8	1	2	590410	920415	33.0	9.260
I	29	CLP	9	2	1	561216	920415	35.3	10.115
I	30	CLP	10	1	1	520331	920415	40.0	10.115
	31	CP	22	1	2	580324	920513	34.1	9.260
I	32	CP	12	1	1	530131	920415	39.2	10.115
I	33	CP	13	2	1	441027	920422	47.5	10.115
I	34	CP	14	1	1	480331	920422	44.1	10.115
I	35	CP	15	2	1			999.0	10.115
I	36	CP	16	1	1	600423	920423	32.0	10.115
I	37	CP	17	2	1	600204	920506	32.3	10.115
I	38	CP	18	1	1	561002	920506	35.6	10.115
I	39	CLP	19	2	1	591011	920506	32.6	10.115
I	40	CLP	20	1	1	550222	920506	37.2	10.115
I	41	CP	21	2	1	570321	920513	35.1	10.115
I	42	CP	22	1	2	580324	920513	34.1	9.260
I	43	CLP	23	2	1	540429	920513	38.0	10.115
I	44	CLP	24	1	1	520118	920513	40.3	10.115
I	45	CL	25	1	1	510525	920513	41.0	10.115
I	46	CL	26	1	1	500405	920513	42.1	10.115
I	47	CP	27	2	1	570103	920527	35.4	10.115
	48	CP	28	1	2	550301	920527	37.2	9.260
	49	CP	29	2	2	560507	920603	36.1	9.260
I	50	CP	30	1	1	550102	920603	37.4	10.115
I	51	CP	31	2	1	641229	920603	27.4	10.115
I	52	CP	32	1	1			999.0	10.115

Record		Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
I	53	CP	33	2	1	610421	920603	31.1	10.115
I	54	CP	34	1	1			999.0	10.115
I	55	CP	35	2	1	510702	920805	41.1	10.115
I	56	CP	36	1	1	520131	920603	40.3	10.115
I	57	CP	37	1	1	561229	920610	35.4	10.115
I	58	CP	38	1	1	530729	920610	38.9	10.115
I	59	CP	39	2	1	461116	920610	45.6	10.115
I	60	CP	40	1	1	480519	920610	44.1	10.115
I	61	CP	41	2	1	540920	920617	37.7	10.115
I	62	CP	42	1	1	540423	920617	38.2	10.115
	63	CL	43	2	2	480409	920624	44.2	9.260
I	64	CL	44	1	1	480202	920624	44.4	10.115
I	65	CP	45	2	1	630110	920624	29.5	10.115
I	66	CP	46	1	1			999.0	10.115
I	67	CLP	47	2	1	430829	920703	48.8	10.115
I	68	CLP	48	1	1	410622	920703	51.0	10.115
	69	CLP	49	2	2	580726	920708	34.0	9.320
I	70	CLP	50	1	1			999.0	10.115
	71	CP	51	2	2	510422	920708	41.2	9.260
I	72	CP	52	1	1	500930	920708	41.8	10.115
I	73	CP	53	2	1		920702	999.0	10.115
I	74	CP	54	1	1			999.0	10.115
I	75	CP	58	2	1	560118	920730	36.5	10.115
I	76	CP	56	1	1	520823	920730	39.9	10.115
I	77	CLP	57	2	1	540719	920812	38.1	10.115
I	78	CLP	58	1	1	470925	920812	44.9	10.115
	79	CLP	59	2	2	510113	920819	41.6	9.260
I	80	CLP	60	1	1	490208	920812	43.5	10.115
	81	CLP	61	2	2	520803	920902	40.1	9.260
I	82	CLP	62	1	1	500706	920902	42.2	10.115
I	83	CLP	63	2	1	610620	920904	31.2	10.115
	84	CLP	64	1	2	591016	920904	32.9	9.260

Record		Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
I	85	CP	66	2	1	510513	920910	41.3	10.115
I	86	CP	66	1	1	580424	920910	34.4	10.115
I	87	CLP	67	2	1	560328	921117	36.6	10.115
I	88	CLP	68	1	1	470712	921117	45.3	10.115
I	89	CP	69	2	1	620204	920930	30.7	10.115
I	90	CP	70	1	1	591123	920930	32.9	10.115
I	91	CL	71	2	1	571107	921005	34.9	10.115
I	92	CL	72	1	1	511027	921005	40.9	10.115
I	93	CP	73	2	1	550328	921013	37.5	10.115
I	94	CP	74	1	1			999.0	10.115
I	95	CP	75	2	1	490223	921014	43.6	10.115
	96	CP	76	1	2	490808	921014	43.2	9.320
	97	CLP	77	2	2	550906	921113	37.2	9.260
I	98	CLP	78	1	1	540114	921113	38.8	10.115
	99	CL	79	2	2	581114	921208	34.1	9.260
	100	CL	80	1	2	570222	921208	35.8	9.320
I	101	CLP	81	2	1	670711	920412	24.8	10.115
I	102	CLP	82	1	1	581007	921204	34.2	10.115
I	103	CL	83	2	1	620129	921209	30.9	10.115
I	104	CL	84	1	1	610407	921209	31.7	10.115
I	105	CL	85	2	1	620210	930222	31.0	10.115
	106	CL	86	1	2	600125	930122	33.0	9.260
I	107	CLP	87	2	1	561208	930127	36.1	10.115
I	108	CLP	88	1	1			999.0	10.115
I	109	CLP	89	2	1	530903	930212	39.4	10.115
I	110	CLP	90	1	1	500310	930212	42.9	10.115
I	111	CLP	91	2	1	481113	930303	44.3	10.115
I	112	CLP	92	1	1	440914	930303	48.5	10.115
I	113	BCL	93	2	1	600611	930318	32.8	10.115
I	114	BCL	94	1	1	511016	930318	41.4	10.115
	115	CP	95	2	2	680829	930414	24.6	9.260
I	116	CP	96	1	1	660216	930414	27.2	10.115

Record		Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
I	117	CLP	97	2	1	531127	930415	39.4	10.115
I	118	CLP	98	1	1	511118	930415	41.4	10.115
I	119	CL	99	2	1	680930	930415	24.5	10.115
	120	BCLP	104	1	2	620901	930513	30.7	9.260
	121	CP	106	1	2	551004	930610	37.7	8.840
I	122	CP	102	1	1	550930	930423	37.6	10.115

A.2.a PARTICIPANTS IN THE LATERAL CEPHALOMETRIC STUDY; CONTROL MALES.

Record	Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
125	1	201	2	1	590102	781108	19.8	8.840
126	1	203	2	1	550801	780301	22.6	8.880
127	1	205	2	1	601012	801126	20.1	8.840
128	1	207	2	1	520916	770606	24.7	8.880
129	1	209	2	1	530509	820107	28.7	8.840
130	1	211	2	1	490608	780908	29.2	8.840
131	1	213	2	1	621007	820127	19.3	8.940
132	1	215	2	1	600204	790830	19.6	8.940
133	1	217	2	1	581217	810116	22.1	8.840
134	1	219	2	1	480722	730613	24.9	8.880
135	1	221	2	1	600902	810608	20.8	8.880
136	1	223	2	1	580914	800428	21.6	8.840
137	1	225	2	1	530618	780202	24.6	8.940
138	1	227	2	1	600724	800613	19.9	8.880
139	1	231	2	1	550923	760923	21.0	8.840
140	1	233	2	1	600507	800826	20.3	8.940
141	1	235	2	1	510723	720323	20.7	8.880
142	1	237	2	1	560621	800829	24.2	8.880
143	1	239	2	1	591211	790626	19.5	8.940
144	2	241	2	1	590831	790618	19.8	8.880
145	2	243	2	1	610614	810929	20.3	8.940
146	2	245	2	1	580726	781109	20.3	8.840
147	2	247	2	1	600618	800701	20.0	8.880
148	2	249	2	1	550505	810428	26.0	8.940
149	2	251	2	1	610617	820114	20.6	8.840
150	2	253	2	1	611116	800418	18.4	8.840
151	2	255	2	1	600722	810714	21.0	8.840
152	2	257	2	1	560117	760206	20.1	8.990
153	2	259	2	1	550210	810831	26.6	8.940
154	2	261	2	1	560225	771125	21.7	8.840
155	2	263	2	1	590613	800218	20.7	8.990

	Record	Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
	156	2	265	2	1	610426	800610	19.1	8.940
	157	3	267	2	1	570310	780123	20.9	8.880
	158	3	269	2	1	530913	801124	27.2	8.880
	159	3	271	2	1	600613	810401	20.8	8.880
	160	3	273	2	1	580127	811106	23.8	8.880
	161	3	275	2	1	510529	720127	20.7	8.940
	162	1	277	2	1	550124	751017	20.7	8.840
	163	4	279	2	1	590726	790129	19.5	8.940
	164	4	281	2	1	610119	810407	20.2	9.050
	165	4	283	2	1	510218	801001	29.6	8.990
	166	4	285	2	1	590226	810210	22.0	8.880
	167	1	229	2	1	380927	610525	22.7	8.840
	168	1	287	2	1	430817	770921	34.1	8.840
	169	2	289	2	1	540113	741003	20.7	8.940
	170	1	291	2	1	560621	810715	25.1	8.990
	171	1	293	2	1	601202	820112	21.1	8.990
	172	1	295	2	1	520706	730508	20.8	8.990
	173	2	297	2	1	600403	810424	21.1	8.840
	174	3	299	2	1	560417	800317	23.9	8.940
I	175	I	301	2	1	380612	900118	51.6	8.880

A.2.b PARTICIPANTS IN THE LATERAL CEPHALOMETRIC STUDY; CONTROL FEMALES.

Record	Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
197	3	200	1	1	500816	790618	28.8	8.840
198	4	202	1	1	390403	610725	22.3	8.840
199	3	204	1	1	540904	721006	18.1	8.840
200	4	206	1	1	540225	760329	22.1	8.840
201	1	208	1	1	520507	720905	20.3	8.840
202	4	210	1	1	370409	781206	41.7	8.840
203	2	212	1	1	551002	781110	23.1	8.840
204	2	214	1	1	451012	820114	36.3	8.840
205	2	216	1	1	540907	721024	18.1	8.840
206	1	218	1	1	381120	610523	22.5	8.840
207	1	220	1	1	550715	741014	19.2	8.840
208	4	222	1	1	540925	761119	22.2	8.840
209	2	224	1	1	550515	730718	18.2	8.840
210	3	226	1	1	510114	720418	21.3	8.940
211	2	228	1	1	551126	821106	26.9	8.840
212	1	230	1	1	341022	610602	26.6	8.840
213	1	232	1	1	390203	610530	22.3	8.840
214	1	234	1	1	500610	700611	20.0	8.840
215	4	236	1	1	530514	740529	21.0	8.840
216	1	238	1	1	560427	800609	24.1	8.840
217	1	240	1	1	681009	901009	22.0	8.840
218	2	242	1	1	520130	731016	21.7	8.840
219	1	244	1	1	390518	610518	22.0	8.840
220	4	248	1	1	570530	801205	23.5	8.840
221	1	250	1	1	701129	900123	19.2	8.880
222	1	252	1	1	381004	620614	23.7	8.840
223	2	254	1	1	520103	720110	20.0	8.940
224	4	256	1	1	390917	610523	21.7	8.840
225	3	258	1	1	470531	710402	23.8	8.840
226	1	260	1	1	560410	790730	23.3	8.840
227	1	262	1	1	530313	780913	25.5	9.200
228	1	264	1	1	651130	850114	19.1	8.840

Record	Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
229	1	266	1	1	610113	810327	20.2	8.840
230	1	268	1	1	521114	740601	21.5	9.200
231	1	270	1	1	521205	791110	26.9	9.290
232	1	272	1	1	530308	810127	27.9	9.200
233	1	274	1	1	530410	781012	25.5	9.200
234	1	276	1	1	530726	790425	25.7	9.200
235	1	278	1	1	530804	790627	25.9	8.840
236	4	248	1	1	560720	810119	24.5	8.840
237	1	280	1	1	530831	850124	31.4	9.200
238	1	282	1	1	530917	750325	21.5	9.200
239	1	284	1	1	531126	750325	21.3	9.200
240	1	286	1	1	540109	780109	24.0	9.200
241	1	288	1	1	540127	741114	20.8	9.200
242	1	290	1	1	540303	780310	24.0	9.200
243	1	292	1	1	540706	850109	30.5	9.200
244	1	294	1	1	550530	791009	24.4	9.200
245	2	296	1	1	530410	780913	25.4	9.200
251	4	210	1	2			999.0	8.840
252	2	212	1	2			999.0	8.840
253	2	214	1	2			999.0	8.840
254	1	218	1	2			999.0	8.840
255	2	224	1	2			999.0	8.840
256	1	227	2	2			999.0	8.880
257	1	231	2	2			999.0	8.840
258	1	237	2	2			999.0	8.880
259	2	243	2	2			999.0	8.940
260	4	246	1	2			999.0	8.840
261	4	248	1	2			999.0	8.840
262	1	250	1	2			999.0	8.880
263	2	261	2	2			999.0	8.840
264	1	262	1	2			999.0	9.200
265	3	267	2	2			999.0	8.880

Record	Group	No.	Sex	Reg.	DOB	DOR	Age	Enlarg.
266	4	283	2	2			999.0	8.990
267	1	286	1	2			999.0	9.200
268	1	287	1	2			999.0	8.840
269	2	296	1	2			999.0	9.200
270	3	299	2	2			999.0	8.940
271	1	239	2	2			999.0	8.940

Record numbers 251-271 represent the records that were randomly chosen for retracing and redigitising.

A.3 PARTICIPANTS IN DNA STUDY: PARENTS AND PROBANDS.

Rec. #	code	Ceph.	Parent	Cleft	Case	Family	BAM	RSA	TAQ
1	1. I.2	3	Yes	CL	21	N	22	12	11
2	1. I.1	4	Yes	CL	12	N	22	12	12
3	1.II..1		No	CL	22	N	22	12	12
4	2. I.2	5	Yes	CLP	18	N	22	22	12
5	2. I.2	6	Yes	CLP	71			22	11
6	3. I.2	7	Yes	CLP	87	Y	22	11	11
7	3. I.1	8	Yes	CLP	88	N		12	12
8	4. I.2	9	Yes	CLP	16	N	12	12	11
9	4. I.1	10	Yes	CLP	27	N	22	22	11
10	4.II.1		No	CLP	28		22	12	11
11	5. I.2	11	Yes	CP	68	Y	12	22	12
12	5. I.1	12	Yes	CP	67	Y	12	12	11
13	5.II.2		No	CP	69	Y	22	22	12
14	6. I.2	13	Yes	CP	1	N	22	22	11
15	6. I.1	14	Yes	CP	0	N			
16	6.II.2		No	CP	70	N	22	22	12
17	7. I.2	17	Yes	CP	24	N	22	22	11
18	7. I.1	18	Yes	CP	23	N	22	22	11
19	7.II.2		No	CP	26	N	22	22	11
20	7.II.1		No	NCP	25	N	22	22	11
21	8. I.2	19	Yes	CLP	86	N	12		12
22	8. I.1	20	Yes	CLP	85	N	22	12	11
23	9. I.2	21	Yes	CP	60	N	22	22	11
24	9. I.1	22	Yes	CP	59	N	22	22	12
25	10. I.2	23	Yes	CLP	74	N	12	12	11
26	10. I.1	24	Yes	CLP	20	N	22	22	11
27	10.II.1		No	CLP	73	N	12	12	11
28	11. I.2	25	Yes	CL	49	N	22	22	12
29	11. I.1	26	Yes	CL	47	N	22	12	11
30	11.II.1		No	CL	48	N	22	22	12
31	12. I.2	27	Yes	CP	5	N	22	22	12

Rec. #	code	Ceph.	Parent	Cleft	Case	Family	BAM	RSA	TAQ
32	12. I.1	28	Yes	CP	2	N	22	11	11
33	13. I.2	29	Yes	CP	80	N	12	12	11
34	13. I.1	30	Yes	CP	62	N	12	12	11
35	13.II.2		No	CP	79	N	12	12	11
36	14.2. I	31	Yes	CP	78	N	12	22	12
37	15. I.2	35	Yes	CP	90		12		11
38	15. I.1	36	Yes	CP	34	-	22	12	11
39	15.II.1		No	CP	35	N	22	12	11
40	16. I.2	41	Yes	CP	33	N	12	12	11
41	16. I.1	42	Yes	CP	32	N	12	12	11
42	17. I.2	43	Yes	CLP	3	N	22	12	11
43	17. I.1	44	Yes	CLP	83	N			
44	17.II.1		No	CLP	75	N	22	12	11
45	18. I.2	45	Yes	CP	57	N	22	11	12
46	18. I.1	46	Yes	CP	0	N			
47	18.II.2.		No	CP	58	N	22	12	11
48	19. I.2	47	Yes	CLP	94		22	22	11
49	19. I.1		No	CLP	93		22	12	
50	20. I.2	51	Yes	CP	40	N	12	12	11
51	20. I.1	52	Yes	CP	0	N			
52	20.II.2		No	CP	41	N	12	21	11
53	21. I.2.	53	Yes	CP	15	N	12	12	11
54	21. I.1	54	Yes	CP	0	N			
55	22. I.2	55	Yes	CP	39	N	12	12	11
56	22. I.1	56	Yes	CP	38	N	12	12	11
57	23. I.2	57	Yes	CLP	63		12	12	11
58	23. I.1	58	Yes	CLP	64		22	22	11
59	23.II.1		No	CLP	10		22	12	11
60	24. I.2	61	Yes	CLP	8		22	12	11
61	24. I.1	62	Yes	CLP	56		22	22	11
62	25. I.2	3	Yes	CLP	30	N	22	22	12
63	25. I.1	64	Yes	CLP	0	N			
64	25.II.1		No	CLP	29	N	22	2	11
65	26. I.2	65	Yes	CP	17	N	12	12	12

Rec. #	code	Ceph.	Parent	Cleft	Case	Family	BAM	RSA	TAQ
66	26. I.1	66	Yes	CP	11	N	22	12	12
67	27. I.2	67	Yes	CLP	19	N	22	22	12
68	27. I.1	68	Yes	CLP	31	N	22	12	11
69	27.II.2		No	CLP	72	N	22	22	12
70	28. I.2	69	Yes	CP	82	N	12	12	11
71	28. I.1	70	Yes	CP	81	Y	22	22	11
72	29. I.2	71	Yes	CL	37	N	22	22	11
73	29. I.1	72	Yes	CL	36	N	22	12	11
74	30. I.2	75	Yes	CP	4	N	22	22	11
75	30. I.1	76	Yes	CP	44	N	12	12	12
76	30.II.1			CP	43	N	22	22	12
77	31. I.2	77	Yes	CLP	55	N	22	11	11
78	31. I.1	78	Yes	CLP	54	N	22	22	22
79	32. I.2	81	Yes	CLP	52		22	12	11
80	32. I.1	82	Yes	CLP	51		22	12	11
81	33. I.2	85	Yes	CL	13	N	22	21	11
82	33. I.1	86	Yes	CL	50	N	22	11	11
83	34. I.2	91	Yes	CLP	45	N	22	11	11
84	34. I.1	92	Yes	CLP	7	Y	22	22	22
85	35. I.2	93	Yes	BCL	62	N	12	12	11
86	35. I.1	94	Yes	BCL	61	N	22	12	12
87	36. I.2	95	Yes	CP	65	Y	22	22	11
88	36. I.1	96	Yes	CP	66	N	22	22	12
89	37. I.2	97	Yes	CLP	92	N	12	22	11
90	37. I.1	98	Yes	CLP	46	Y	22	22	11
91	38. I.2	101	Yes	CP	77	N	22	22	11
92	38. I.1	102	Yes	CP	7	N			11
93	39. I.2	103	Yes	BCLP	53	N	22	22	11
94	39. I.1	104	Yes	BCLP	9	N	22	12	11
95	3.II.1		No	CLP	89	N		12	11
96	8.II.1		No	CLP	84		22	22	

A.4 PARTICIPANTS IN DNA STUDY: CONTROL

DNA No.	*Rsa*I	*Bam*HI	*Taq*I
4389	B1B1	A2A2	C1C1
4443	B2B2	A2A2	C1C2
4582	B1B1	A1A2	C1C1
4583	B2B2	A2A2	C1C1
4726	B2B2	A2A2	C1C1
4736	B2B2	A2A2	C1C1
4839	B2B2	A2A2	C1C1
4740	B1B1	A2A2	C1C2
4742	B1B1	A2A2	C1C1
4743	B1B1	A1A2	C1C1
4843	B1B1	A2A2	C1C1
4844	B2B2	A2A2	C1C1
4880	B2B2	A2A2	C1C1
4881	B2B2	A2A2	C1C2
4888	B2B2	A2A2	C1C1
4889	B1B2	A2A2	C1C1
4892	B2B2	A2A2	C1C1
4910	B1B2	A2A2	C1C1
4911	B2B2	A2A2	C1C1
5021	B2B2	A2A2	C1C1
5022	B2B2	A2A2	C1C1
5034	B2B2	A2A2	C1C1
5035	B1B2	A1A2	C1C1
5036	B2B2	A2A2	C1C1
5037	B1B2	A1A2	C1C1
5141	B1B2	A2A2	C1C1
5142	B1B2	A1A2	C1C1
5185	B1B2	A2A2	C1C1
5186	B2B2	A2A2	C1C1
5607	B2B2	A2A2	C1C1
5893	B1B1	A1A2	C1C1
5925	B1B2	A2A2	C1C1

DNA No.	*Rsa*I	*Bam*HI	*Taq*I
5926	B2B2	A2A2	C1C1
5933	B1B2	A1A2	C1C1
5936	B2B2	A2A2	C1C1
5938	B1B1	A1A2	C1C1
5973	B1B2	A1A2	C1C1
5975	NR	A2A2	C1C1
6006	B1B2	A2A2	C1C1
6007	B1B2	A1A2	C1C1
6015	B2B2	A2A2	C1C2
6016	B2B2	A2A2	C1C1
6019	NR	A2A2	NR
6020	B1B2	A2A2	NR
6028	B1B1	A2A2	C1C1
6029	B1B1	A2A2	C1C1
001	B1B2	A1A2	C1C1
003	B2B2	A2A2	C1C2
004	B1B2	A2A2	C1C1
005	B2B2	A2A2	C1C1
006	B2B2	A2A2	C1C1
008	B1B2	A1A2	C1C1
009	B1B2	A2A2	C1C1
010	B2B2	A2A2	C1C1
012	B1B2	A2A2	C1C1
013	B2B2	A2A2	C1C1
014	B1B2	A2A2	C1C1
015	B2B2	A2A2	C1C1
016	B1B2	A2A2	C1C1
017	B1B2	A1A2	C1C1
018	B1B2	A1A2	C1C1
119	B1B2	A1A2	C1C1

NR = No Result

APPENDIX B

DEFINITIONS OF 136 CEPHALOMETRIC POINTS

B.1 DEFINITION OF POINTS

Those point definitions referred to in previous publications, many of which are recognised homologous landmarks, are labelled as such. There are also intermediate derived and constructed points unique to this study which were created by the author to enable description of desired areas and parameters.(see section 2.3.3.). The landmark names and definitions for the 99 points used in the mandibular, maxillary, cranial base and nasal bone analysis and the 37 points used in the cranial analysis are as follows:

A. Mandibular landmark definitions

1. Point 1: the lingual contact of alveolar bone with the mandibular central incisor. This will often correspond with the amelocemental junction (1) (Riolo et al., 1974)
2. Point 2: an intermediate point on the outline of the superior border of the mandibular alveolar ridge midway between the alveolar margin lingual to the lower central incisor and the alveolar margin mesial to the lower first molar. (2)
3. Point 3: the mesial contact of alveolar bone with the mandibular first permanent molar. The distal contact of alveolar bone with the second deciduous molar is marked in the absence of the first permanent molar. (3) (Riolo et al., 1974)
4. Point 4: an intermediate point on the outline of the superior border of the alveolar margin distal and superior to the alveolar margin distal to the lower first molar. (4)
5. Point 5: an intermediate point on the outline of the anterior border of the ramus inferior to R1. (5)
6. R1: the deepest point on the anterior border of the ramus located halfway between the superior and inferior curves on a tangent to the pterygoid vertical line and perpendicular to the Frankfort horizontal plane. (R1) (Ricketts et al., 1982)
7. Point 6: the most convex point on the outline of the anterior border of the ramus superior to R1. (6)
8. Point 7: the most superior point on the average of the right and left outlines of the coronoid processes. (7) (Riolo et al., 1974)
9. Point 8: an intermediate point on the outline of the coronoid process posterior and inferior to the tip of the coronoid process. (8)

10. R3: deepest point on the sigmoid notch halfway between the anterior and posterior curves and linked to the Frankfort plane and the Pterygoid vertical line (R3) (Ricketts et al., 1982)
11. Point 9: an intermediate point on the outline of the sigmoid notch posterior and superior to R3. (9)
12. Point 10: the point of intersection of the inferior surface of the cranial base and the averaged anterior surfaces of the mandibular condyles. (10) (Riolo et al., 1974)
13. Point 11: the point of maximum convexity on the medial surface of the averaged images of the mandibular condyles. (11)
14. Condylion: the most posterior superior point on the curvature of the average of the right and left outlines of the condylar heads. This is a mid axial point determined by a perpendicular to lines constructed from the inner and outer surfaces of the condylar head. (Cd) (Riolo et al., 1974)
15. Point 12: the tangent point on the posterior surface of the average image of the mandibular condyles drawn from Rtan. (12)
16. Articulare: The point of intersection of the inferior contour of the clivus and the averaged posterior surfaces of the mandibular condyles. (Ar) (Bjork, 1947)
17. Point 13: an intermediate point on the outline of the posterior border of the ramus between the articulare and R2. (13)
18. R2: located on the posterior border of the ramus opposite R1 and halfway between the superior and inferior curves (R2) (Ricketts et al., 1982)
19. Rtan: obtained by constructing a tangent to the posterior border of the ramus superior to gonion and contacting the posterior border of the mandibular condyle. (Rtan) (Ricketts et al., 1982)
20. Gonion: the midpoint of the angle of the mandible found by bisecting the angle formed by the mandibular plane and a plane through articulare forming a tangent to the posterior border of the ramus. (Go) (Riolo et al., 1974)
21. Point 14: Tangent point to lower border of the mandible drawn from menton, and posterior point of the mandibular plane. (14)

22. R4: located on the inferior border of the ramus halfway between the anterior and posterior curves and opposite R3. (R4) (Ricketts et al., 1982)
23. Point 15: an intermediate point on the outline of the lower border of the mandible approximately twenty percent of the distance from R4 to menton. (15)
24. Point 16: an intermediate point on the outline of the lower border of the mandible approximately forty percent of the distance from R4 to menton. (16)
25. Point 17: an intermediate point on the outline of the lower border of the mandible approximately sixty percent of the distance from R4 to menton. (17)
26. Point 18: an intermediate point on the outline of the lower border of the mandible approximately eighty percent of the distance from R4 to menton. (18)
27. Menton(1): the most inferior point on the symphyseal outline recorded for the first time. (Me1) (Riolo et al., 1974)
28. Point 19: point on the lingual aspect of the symphyseal outline approximately halfway between menton and genion (19)
29. Genion: the point of maximum posterior curvature on the lingual surface of the symphysis. (Ge) (Riolo et al., 1974)
30. Point 20: an intermediate point on the outline of the inner border of the symphysis midway between genion and the point on the alveolar margin lingual to the lower central incisor. (20)
31. Point 21: the lingual contact of alveolar bone with the mandibular central incisor digitised for the second time. (21)
32. Infradentale: the anterior superior point on the mandible at its labial contact with the mandibular central incisor. (Id) (Riolo et al., 1974)
33. B point: the point most posterior to a line from infradentale to pogonion on the anterior surface of the symphyseal outline of the mandible and should lie adjacent to the apical third of the lower incisor root. (B) (Riolo et al., 1974)
34. pm point: the point on the anterior border of the symphysis where the curvature changes from concave to convex. (pm) (Ricketts et al., 1982)
35. Pogonion: the most anterior point on the outline of the chin determined by taking a tangent through nasion. (Pog) (Riolo et al., 1974)

36. Gnathion: the most anterior inferior point on the contour of the symphysis determined by bisecting the angle formed by the mandibular plane and a line passing through nasion and pogonion. (Gn) (Riolo et al., 1974)
37. Menton(2): the most inferior point on the symphyseal outline having the same definition as menton(1) but digitised on a second occasion. (Me2) (Riolo et al., 1974)
38. Incisal tip of lower incisor: the incisal tip of the mandibular central incisor. (Ili) (Riolo et al., 1974)
39. Apex of lower incisor: the root tip of the mandibular central incisor. When this has not fully formed the mid point of the growing root tip is marked (Ali) (Riolo et al., 1974)
40. Xi point: Constructed point defined as the intersection of the diagonals of a rectangle constructed through points R1, R2, R3 and R4. (Ricketts et al., 1982)

Maxillary landmark definitions

41. Supradentale: the most anterior inferior point on the maxilla on its labial contact with the maxillary central incisor. (Sd) (Riolo et al., 1974)
42. A point: the most posterior point on the curve of the maxilla between the anterior nasal spine and supradentale. (A) (Riolo et al., 1974)
43. Point 22: an intermediate point which marks the point of maximum concavity on the maxillary outline between A point and ANS. (22)
44. Anterior Nasal Spine: sharp median process formed by the forward prolongation of the two maxillae at the lower margin of the anterior aperture of the nose. (ANS) (Riolo et al., 1974)
45. Point 23: the point where the lateral wall of the piriform aperture intersects with the nasal floor. (23)
46. Point 24: the most superior point on the profile of the nasal floor formed by the projected images of the lateral walls of the piriform apertures. (24)
47. Point 25: The point of maximum concavity on the profile of the nasal floor between points 24 and 26. (25)

48. Point 26: An intermediate point on the outline of the nasal floor approximately midway between points 24 and 27. (26)
49. Point 27: The point of intersection of a line running parallel to the PMV line through KR point with the outline of the nasal floor. (27)
50. Posterior Nasal spine: Process formed by the united projecting medial ends of the posterior borders of the two palatine bones. (PNS) (Riolo et al., 1974)
51. Point 28: An intermediate point on the bony contour of the oral surface of the hard palate approximately midway between PNS and point 29. (28)
52. Point 29: The point of intersection of a line running parallel to the PMV line through KR point with the outline of the oral surface of the hard palate. (29)
53. Point 30: An intermediate point on the bony contour of the oral surface of the hard palate approximately midway between points 29 and 31. (30)
54. Point 31: The point of maximum concavity on the antero-superior bony contour of the oral surface of the hard palate. (31)
55. Point 32: The lingual contact of alveolar bone with the maxillary central incisor. The point generally corresponds with the lingual cemento-enamel junction. (32) (Riolo et al., 1974)
56. Incisal tip of upper incisor: the incisal tip of the more prominent maxillary central incisor. (UIe) (Riolo et al., 1974)
57. Apex of upper incisor: the root tip of the maxillary central incisor. (UIa) (Riolo et al., 1974)
58. Upper molar mesial cusp tip: the anterior cusp tip of the maxillary first molar (UMT) (Riolo et al., 1974)
59. Anterior point of Downs occlusal plane: the midpoint of the line connecting ILi and UIe which represents the anterior point through which Downs occlusal plane passes. (Riolo et al., 1974)
60. Sphenoethmoid: junction between the jugum sphenoidale (ie the sphenoid bone outline anterior to the hypophyseal fossa) and the averaged greater sphenoid wing. (Se) (Riolo et al., 1974)

61. Point 33: the point of maximum convexity on the antero-superior outline of the anterior wall of the sphenoid bone. (33)
62. Point 34: the point of greatest convexity on the antero-inferior outline of the anterior wall of the sphenoid bone. (34)
63. Point 35: the point of intersection of the Frankfort plane with the outline of the ventral surface of the sphenoid bone. (35)
64. Point 36: the point of the greatest convexity between points 35 and 37 on the ventral surface of the sphenoid bone. (36)
65. Point 37: the point of intersection of the anterior border of the mandibular condylar process with the outline of the ventral surface of the basi-occipital bone. (37)
66. Articulare: digitised for the second time. (Ar2)
67. Point 38: the point of greatest concavity on the ventral surface of the basilar part of the occipital bone between articulare and basion. (38)
68. Basion: the most inferior posterior point on the anterior margin of foramen magnum. (Ba) (Riolo et al., 1974)
69. Point 39: The point of intersection of a line drawn from nasion to opisthion with the outline of the dorsal aspect of the basi-occipital bone. (39)
70. Point 40: the point of intersection of the Frankfort Plane (drawn from Orbitale to Porion) with the outline of the dorsal aspect of the basi-occipital bone. (4O)
71. Point 41: the point of intersection of 9 line from nasion drawn through U point with the superior contour of the clivus. (41)
72. Point 42: the point of maximum concavity on the dorsal aspect of the outline of the posterior clinoid process between point 41 and dorsum sellae. (42)
73. Point 43: the point of greatest convexity on the posterior aspect of the posterior clinoid process. (43)
74. Dorsum sellae: the most anterior superior point on the posterior wall of the sella turcica. (Ds) (Walker and Kowalski, 1971)
75. Point 44: the point of intersection of the SN line with the posterior wall of the sella turcica. (44)

76. Point 45. the point of greatest concavity on the internal outline of the sella turcica between point 44 and U point. (45)
77. U point: The lowest point in the outline of the hypophyseal fossa identified by a tangent line drawn from nasion. (U) (Nakasima et al., 1982)
78. Point 46: the point of greatest concavity on the internal outline of the sella turcica between U point and Point 47. (46)
79. Point 47: the point of intersection of the SN line with the anterior wall of the sella turcica. (47)
80. Tuberculum sellae: the most posterior superior point on the anterior wall of the sella turcica (Ts). (Walker and Kowalski, 1971)
81. Point 48: the point of greatest convexity on the jugum sphenoidale between tuberculum sellae and sphenoethmoid (Se). (48)
82. Point 49: the point of greatest concavity on the jugum sphenoidale between points tuberculum sellae and sphenoethmoid. (49)
83. Sphenoethmoid: digitised for the second time (Se2).
84. Frontomaxillary nasal suture: the junction of the frontal maxillary and nasal bones. (FMN) (Riolo et al., 1974)
85. Point 50: point on the inferior outline of the nasal bones approximately 25% of the distance from the frontomaxillary nasal suture to rhinion (R). (50)
86. Point 51: point on the inferior outline of the nasal bones approximately 50% of the distance from the frontomaxillary nasal suture to rhinion. (51)
87. Point 52: point on the inferior outline of the nasal bones approximately 75% of the distance from the frontomaxillary nasal suture to rhinion. (52)
88. Rhinion: the radiographic tip of the right and left nasal bones in the mid-saggital plane. (R) (Walker and Kowalski, 1971)
89. Point 53: a point on the superior outline of the nasal bones approximately one third of the distance from rhinion to nasion. (53)
90. Point 54: a point on the superior outline of the nasal bones approximately two thirds of the distance from rhinion to nasion. (54)

91. Nasion: radiographic projection of the junction of nasal and frontal bones at the anterior end of the midsaggital plane. (N) (Riolo et al., 1974)
92. Sella: the centre of the bony crypt forming the sella turcica. (S) (Riolo et al., 1974)
93. Orbitale: the lowest point on the average of the right and left borders of the bony orbit. (Or) (Riolo et al., 1974)
94. Porion: the midpoint of the line connecting the most superior point of the radiopacity generated by each of the two ear rods of the cephalostat. (Po) (Riolo et al., 1974)
95. Point 55: the point of intersection on the posterior nasopharyngeal wall of a line drawn from posterior nasal spine to basion. (55)
96. Point 56: Projected point identified by the intersection of a line passing through condylion parallel to the Frankfort plane, with a perpendicular to this line dropped vertically from sella. (56)
97. Hormion: Point of intersection between the averaged radiographic image of the posterior borders of the right and left pterygoid plates and the inferior border of the cranial base. (Hor) (Walker and Kowalski, 1971).
98. Key Ridge point: the most anterior inferior point on the averaged radiographic image of the right and left maxillary key ridges. (KR) (Walker and Kowalski, 1971)
99. Mid palatal point: point half way between point 27 and point 29 on the superior and inferior borders of the maxillary bone respectively. (MPP)

The landmark names and definitions for cranial vault are as follows:

1. Nasion: radiographic projection of the junction of nasal and frontal bones at the anterior end of the midsaggital plane, digitised for the purpose of the cranial analysis. (N2)
2. C1: Point of maximum convexity on the external cranial outline approximately one third of the distance between nasion and glabella. (C1)

3. C2: point of maximum convexity on the external cranial outline approximately two thirds of the distance between nasion and glabella. (C2)
4. Glabella: The most anterior point on the outline of the frontal bone in the midsaggital plane determined by a perpendicular extended from SN line. (Gla) (Brown, 1973)
5. C3: point of maximum convexity on the external cranial outline between glabella and C4. (C3)
6. C4: point of maximum convexity on the external cranial outline between C3 and C5. (C4)
7. C5: point of maximum convexity on the external cranial outline between nasion and bregma identified by the subtenuce perpendicular to the Frontal cord. (C5)
8. C6: point of maximum convexity on the external cranial outline between C5 and C7. (C6)
9. C7: point of maximum convexity on the external cranial outline between bregma and vertex. (C7)
10. C8: point of maximum convexity/concavity on the external cranial outline between C7 and bregma. (C8)
11. Bregma: the exocranial intersection of the coronal and saggital sutures. (Br) (Brown, 1973)
12. C9: point of maximum convexity/concavity on the external cranial outline between bregma and vertex and approximately half way between bregma and vertex. (C9)
13. Vertex: the most superior point on the outline of the parietal bones of the cranium. (V) (Brown, 1973)
14. C10: point of maximum convexity on the external cranial outline between vertex and C11. (C10)
15. C11: point of maximum convexity on the external cranial outline between bregma and lambda identified by the subtenuce perpendicular to the parietal cord. (C11)
16. C12: point of maximum convexity on the external cranial outline between C11 and C13 and approximately one quarter of the distance between C11 and lambda (C12)

17. C13: point of maximum convexity on the external cranial outline between C12 and C14 and approximately half way between C11 and lambda. (C13)
18. C14: point of maximum convexity on the external cranial outline between C13 and lambda and approximately three quarters of the distance between C11 and lambda. (C14)
19. Lambda: the exocranial intersection of the saggital and lambdoid sutures. (L) (Brown, 1973)
20. C15: point of maximum convexity on the external cranial outline between L and C16 and approximately one third of the distance between lamda and opisthocranion (CPo). (C15)
21. C16: point of maximum convexity on the external cranial outline between C15 and opisthocranion and approximately two thirds of the distance between lambda and opisthocranion. (C16)
22. Opisthocranion: the most posterior point on the outline of the occipital bone in the midsaggital plane, determined by a perpendicular to the SN line. (CPo) (Brown, 1973)
23. C17: point of maximum convexity on the external cranial outline between CPo and C18 and approximately one quarter of the distance between opisthocranion and CNS. (C17)
24. C18: point of maximum convexity on the external cranial outline between C17 and C19 and approximately halfway between opisthocranion and CNS. (C18)
25. C19: point of maximum convexity on the external cranial outline between C18 and CNS and approximately three quarters of the distance between opisthocranion and CNS. (C19)
26. CNS: point on the external cranial outline intersected by a line 180° from the SN line. (CNS)
27. C20: point of maximum convexity on the external cranial outline between lambda and opisthion identified by the subtenuce perpendicular to the occipital cord. (C20)

28. C21: point of maximum concavity on the external cranial outline between C20 and C22. (C28)
29. C22: point of maximum convexity on the external cranial outline between C21 and C23 and approximately one quarter of the distance between C20 and opisthion. (C22)
30. C23: point of greatest convexity on the postero-inferior surface of the occipital bone between C22 and C24. (C30)
31. C24: point of greatest convexity on the inferior surface of the occipital bone between C23 and opisthion. (C31)
32. Opisthion: the posterior midsaggital point on the posterior margin of foramen magnum. (Op) Riolo et al., 1974.
33. Basion: the lowest point on the external surface of the anterior margin of foramen magnum in its' midsaggital plane. (Ba) (Brown, 1973)
34. Sella: Visually adjudged centre point of the hypophyseal fossa. (S) (Riolo et al., 1974)
35. N2: Nasion digitised for a second time. (N2)
36. Orbitale: the lowest point on the average of the right and left borders of the bony orbit. (Or) (Riolo et al., 1974.)
37. Porion: The midpoint of the line connecting the most superior point of the radiopacity generated by each of the two ear rods of the cephalostat. (Po) (Riolo et al., 1974.)

B.2 PARAMETERS MEASURED

1) Mandibular ramus, body and symphysis.

(a) AREA and LINEAR

s1.	Area contained by the symphyseal outline.	
s2.	Total cross-sectional area of ramus and body as viewed on a lateral cephalogram.	
s3.	Rtan-Gn	Mandibular body length.
s4.	R1-R2	Ramus width (Ricketts *et al.* 1982).
s5.	R3-R4	Ramus height (Ricketts *et al.* 1982).
s6.	Pog-Ge	Symphyseal width.
s7.	Cd-Gn	Mandibular length.
s8.	Cd-14	Mandibular ramus height.
s9.	Id-Me	Anterior lower dental height.
s10.	3 to Go-Me	Posterior lower dental height.

(b) ANGULAR

s11.	Ar-Rtan to Me-14	Gonial angle.
s12.	Cd-Xi-pm	Xi angle (Ricketts *et al.*, 1982).
s13.	$\overline{1}$-Go-Me	Lower incisor to mandibular plane.

2) Facial Measurements (including Maxilla)

(a) AREA and LINEAR (maxilla)

s14.	Sd-32 as	Radiographic cross-sectional area of palatal outline viewed on a lateral cephalogram.
s15.	ANS-PNS	Maxillary length.
s16.	26-ANS	Palatal length anterior to key ridge.
s17.	PNS-26	Palatal length posterior to key ridge.
s18.	Or-MPP	Orbital to palatal distance.
s19.	KR-MPP	Key ridge to palatal distance.
s20.	ANS-Sd	Anterior upper dental height.

(b) ANGULAR (maxilla)

s21.	N-ANS-PNS	Anterior position and inclination of palate.
s22.	$\underline{1}$-PP	Upper incisor to palatal plane.
s23.	OP-PP	Occlusal plane to palatal plane.

(a) LINEAR (facial)

s24.	N-Me	Anterior total face height.
s25.	N-ANS	Upper anterior face height.
s26.	Se-PNS	Posterior upper face height.
s27.	S-Gn	Sella to gnathion.
s28.	S-Go	Posterior total face height.
s29.	PNS-55	Soft tissue naso-pharyngeal depth.
s30.	PNS-HOR	Bony nasopharyngeal depth.
s31.	N-Me/ANS-PNS	Anterior face height ratio (UFH : LFH).

(b) ANGULAR (facial)

s32.	SNA	Maxillary position relative to Cranial Base.
s33.	SNB	Mandibular position relative to Cranial Base.
s34.	ANB	Antero-posterior skeletal discrepancy.
s35.	MMPA	Maxillary mandibular planes angle.
s36.	N-S to Go-Me	SN plane to mandibular plane.
s37.	N-S-Rtan-Ar	Inclination of ramus to anterior cranial base.
s38.	S-N-ANS	Maxillary prominence measured to ANS.
s39.	Or-N-S	Orbital position relative to cranial base.
s40.	N-S to Or-Po	Cranial base/Frankfort plane angle.

3) Cranial Base Measurements (including Nasal)

(a) AREA and LINEAR (Cranial)

s41.	Area contained by Cranial base outline on a lateral cephalogram.	
s42.	S-N	Anterior cranial base length.
s43.	N-Ba	Cranial base length.
s44.	S-Ba	Clivus length.

s45. N-Se Anterior cranial base length measured from nasion to sphenoethmoid.

s46. Se-S Length of jugum spenoidale.

s47. 44-47 Sella width.

s48. N-S-Ba Cranial base angle.

s49. Cd-56 Sella-condylion distance in horizontal plane.

s50. S-56 Sella - condylion distance in vertical plane.

(a) AREA and LINEAR (nasal)

s51. Area contained within the outline of the nasal bones.

s52. N-R Nasion to rhinion.

s53. R-A Rhinion to A point.

s54. R-Or Rhinion to orbitale.

(b) ANGULAR (nasal)

s55. S-N-R Nasal bones to cranial base.

s56. N-R-A Nasal bones to maxilla.

s57. N-R-Or Nasal bones to orbitale.

4) Cranial Vault Measurements

c1. N-Br Chord length of frontal bone.

c2. Br-L Chord length of parietal bone.

c3. L-Op Chord length of occipital bone.

c4. V-Ba Cranial height

c5. Gla-CPo Cranial width

c6. Cross-sectional area of the cranial vault as projected onto a lateral cephalogram as defined by N, C1...C23, OP, Ba, S, N.

c7. Frontal subtenuce measured from c5

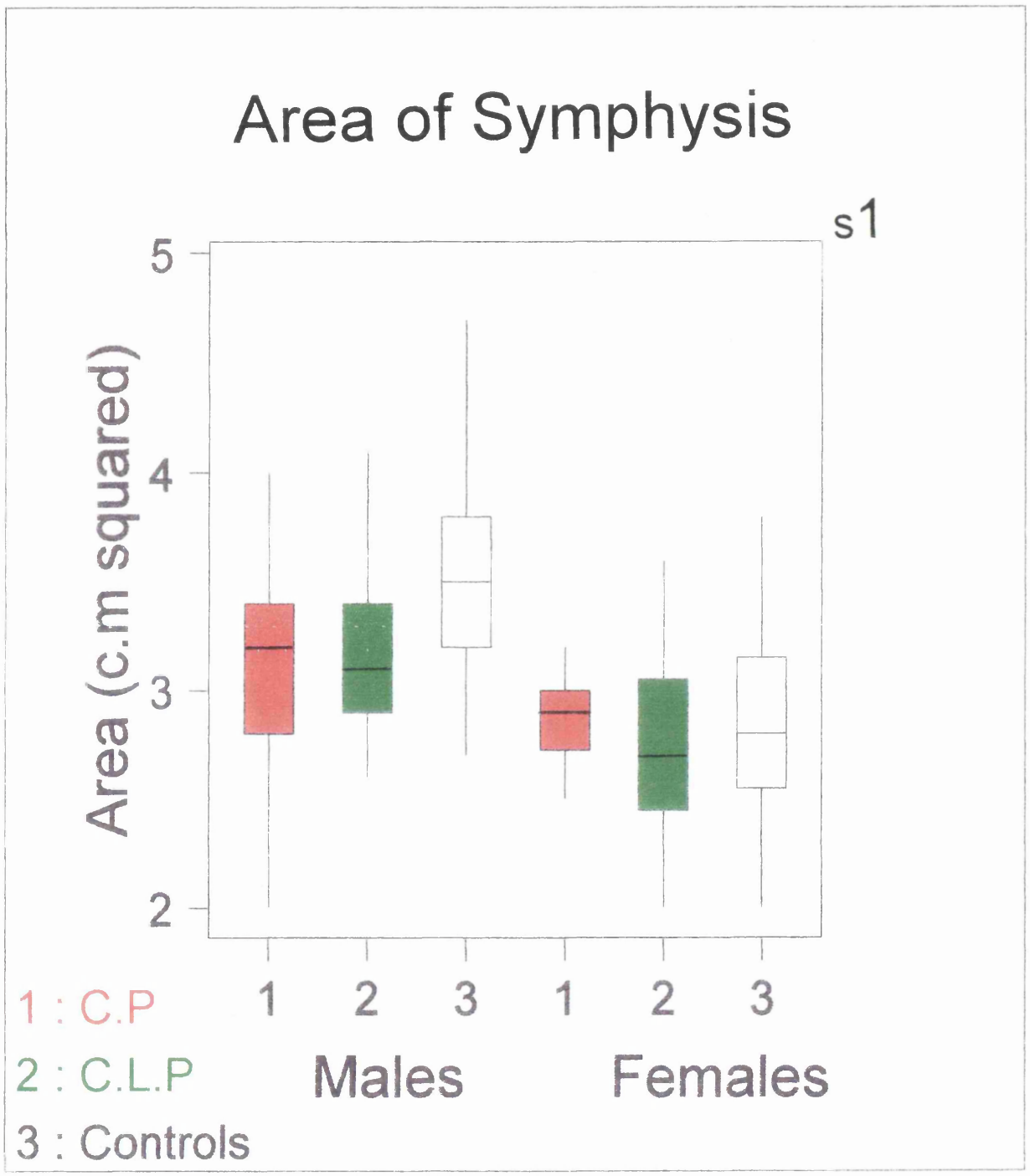
c8. Parietal subtenuce measured from c11

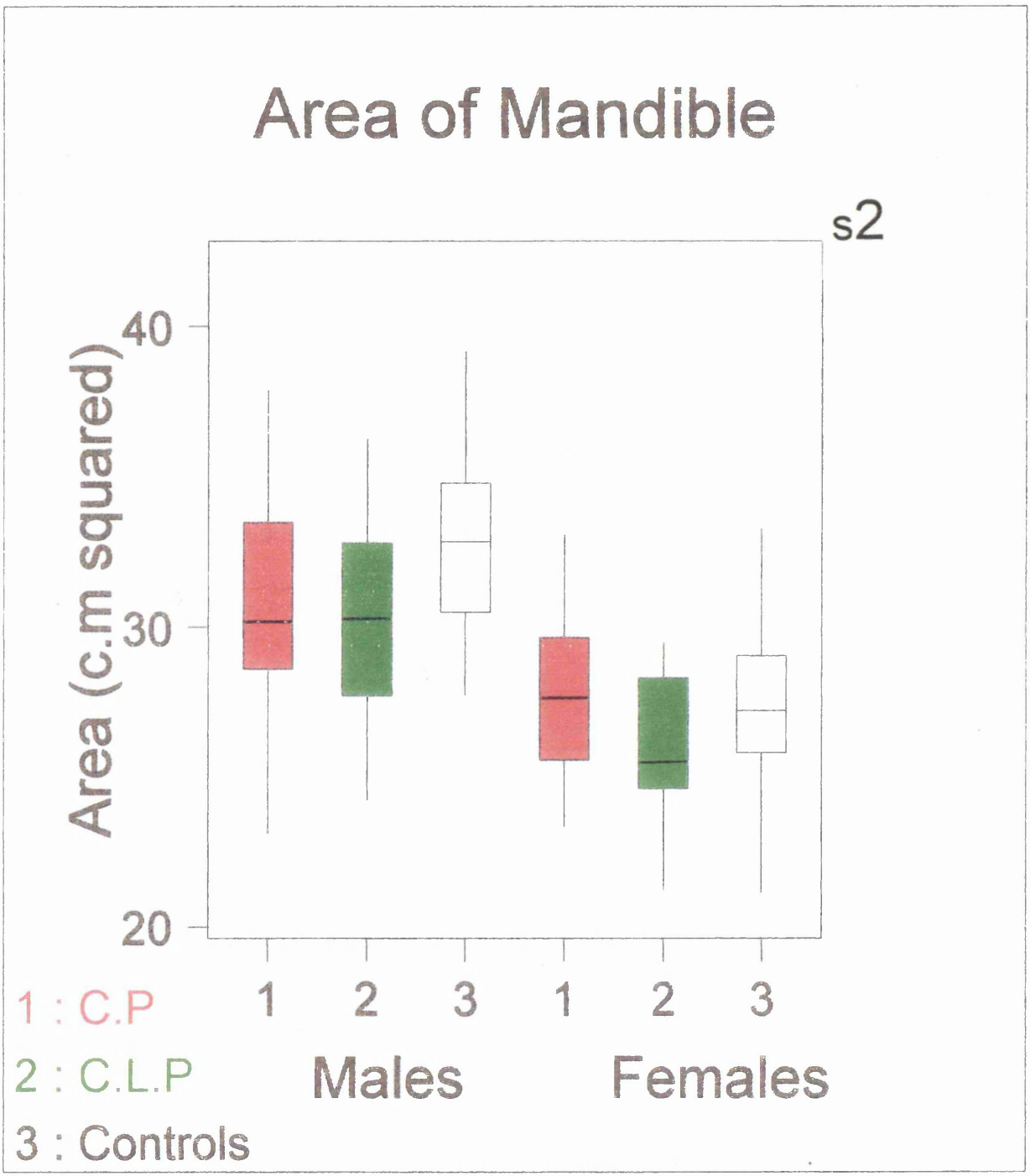
c9/c10. Occipital subtenuce measured from c19 or c20

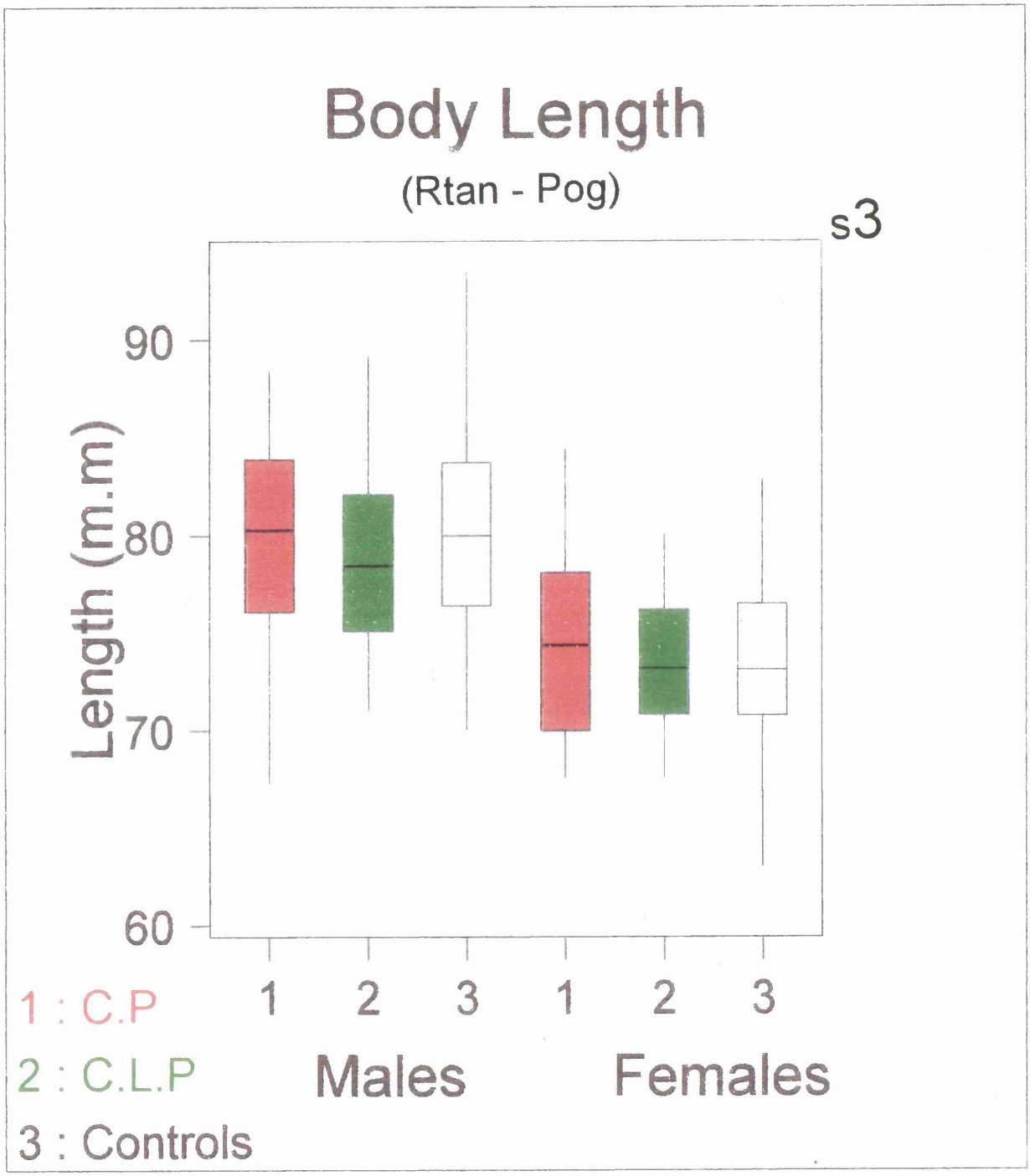
APPENDIX C

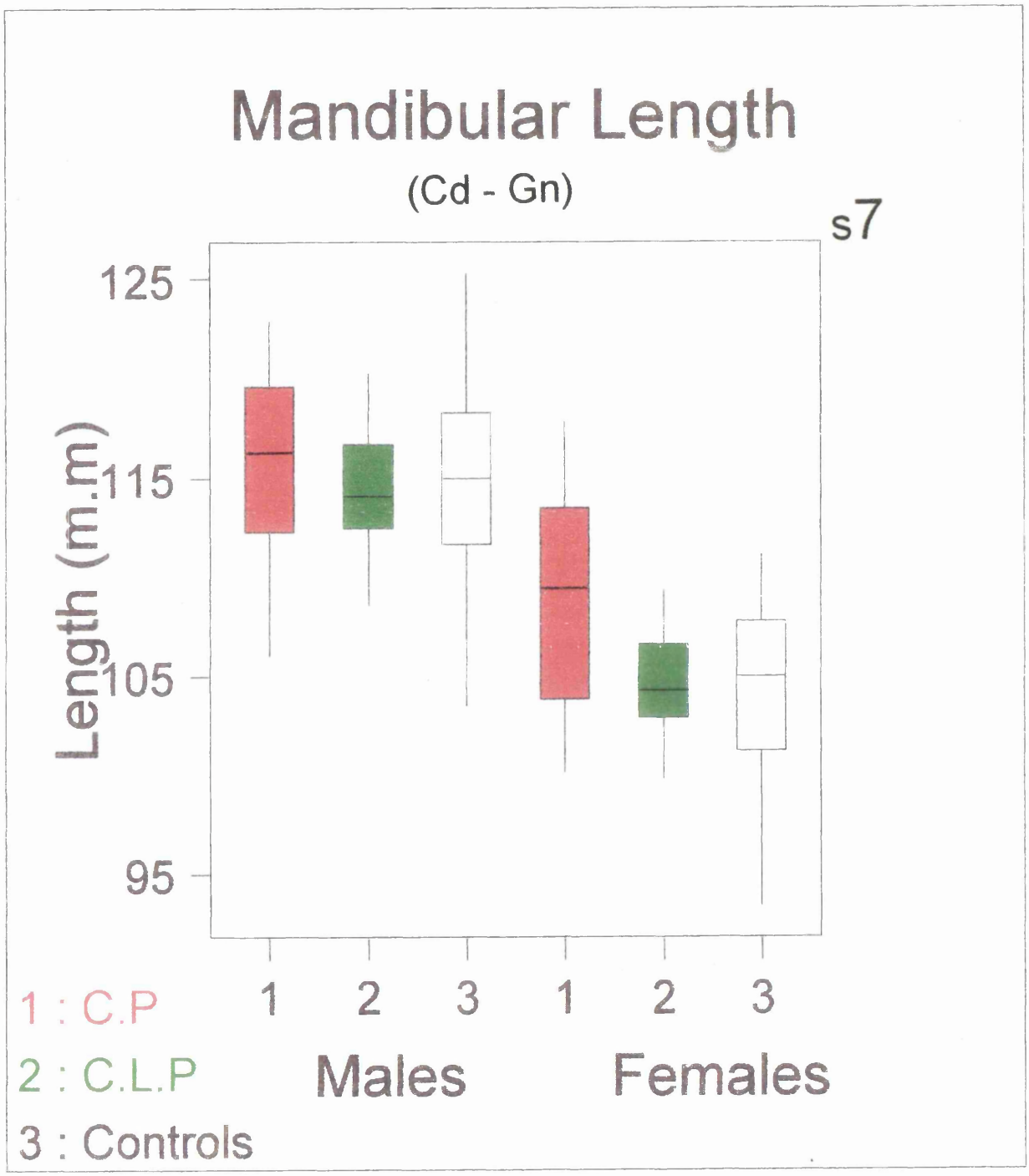
COMPARISON OF 37 CRANIOFACIAL VARIABLES BETWEEN THE PARENTS OF CLEFT CHILDREN AND THE CONTROLS, ANALYSED SEPARATELY FOR MALES AND FEMALES.

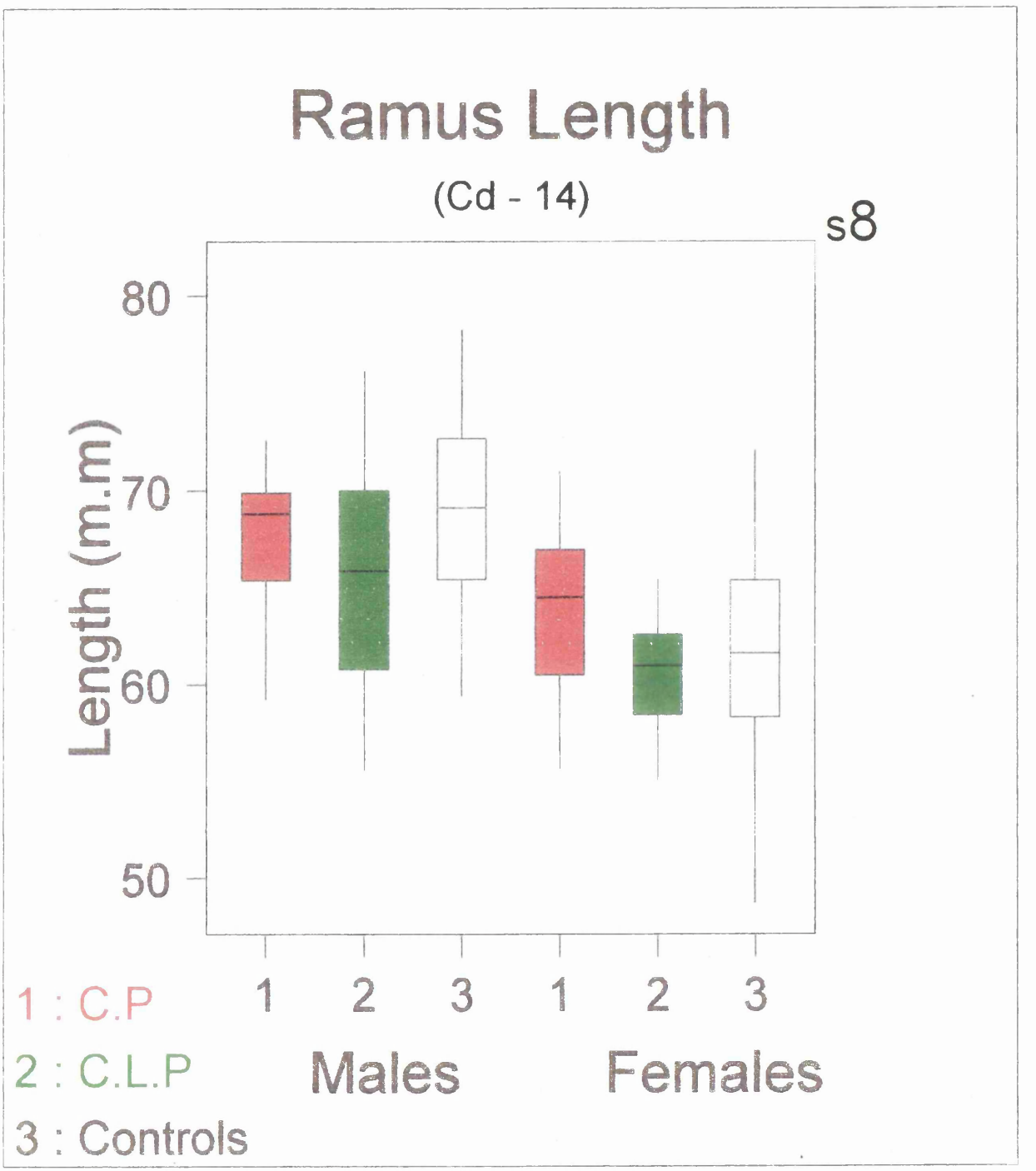
		Males	Females
CP	n =	18	17
CLP	n =	23	25
Control	n =	49	50

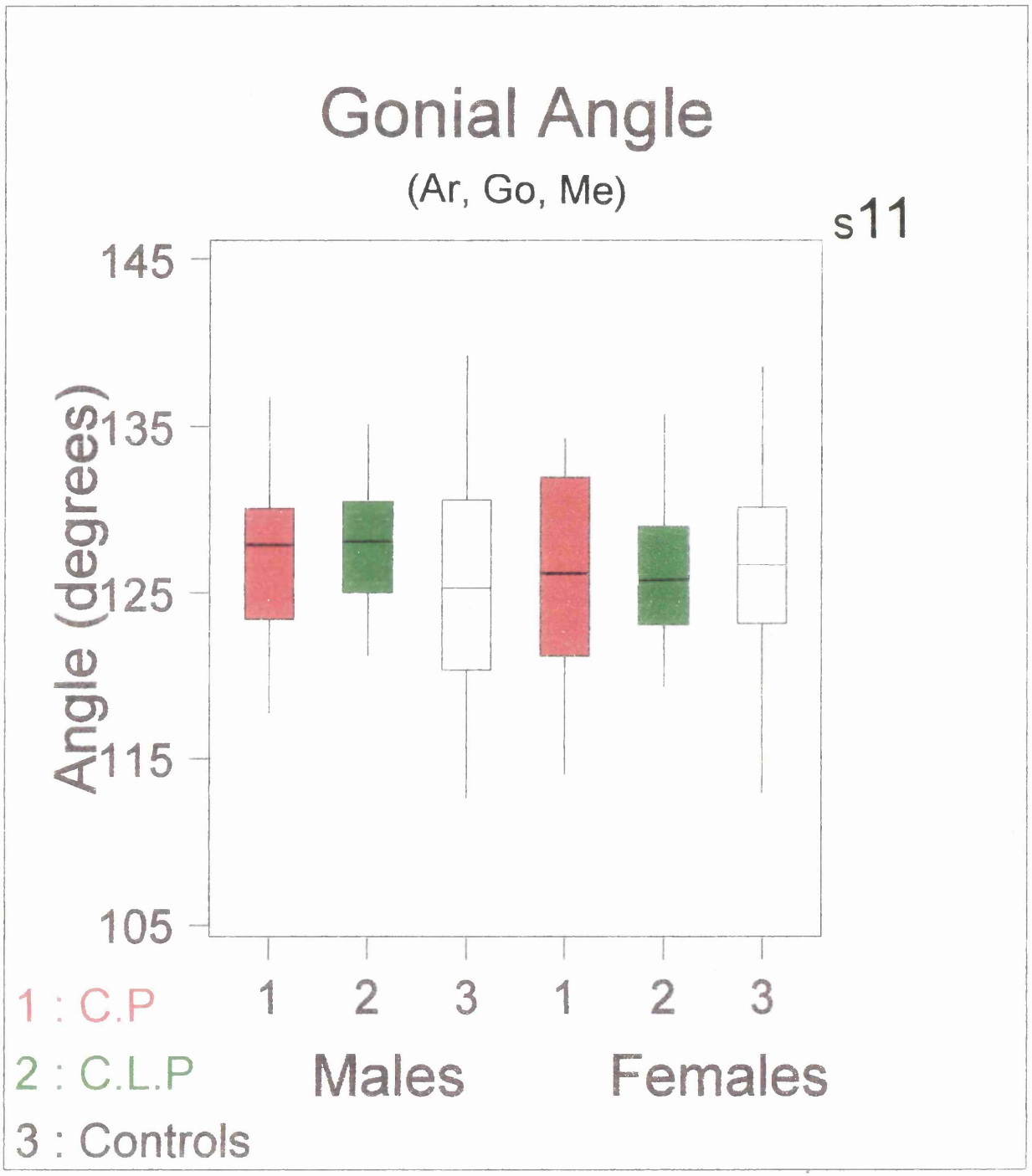

Area of Symphysis
s1
Area (c.m squared)
5
4
3
2
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

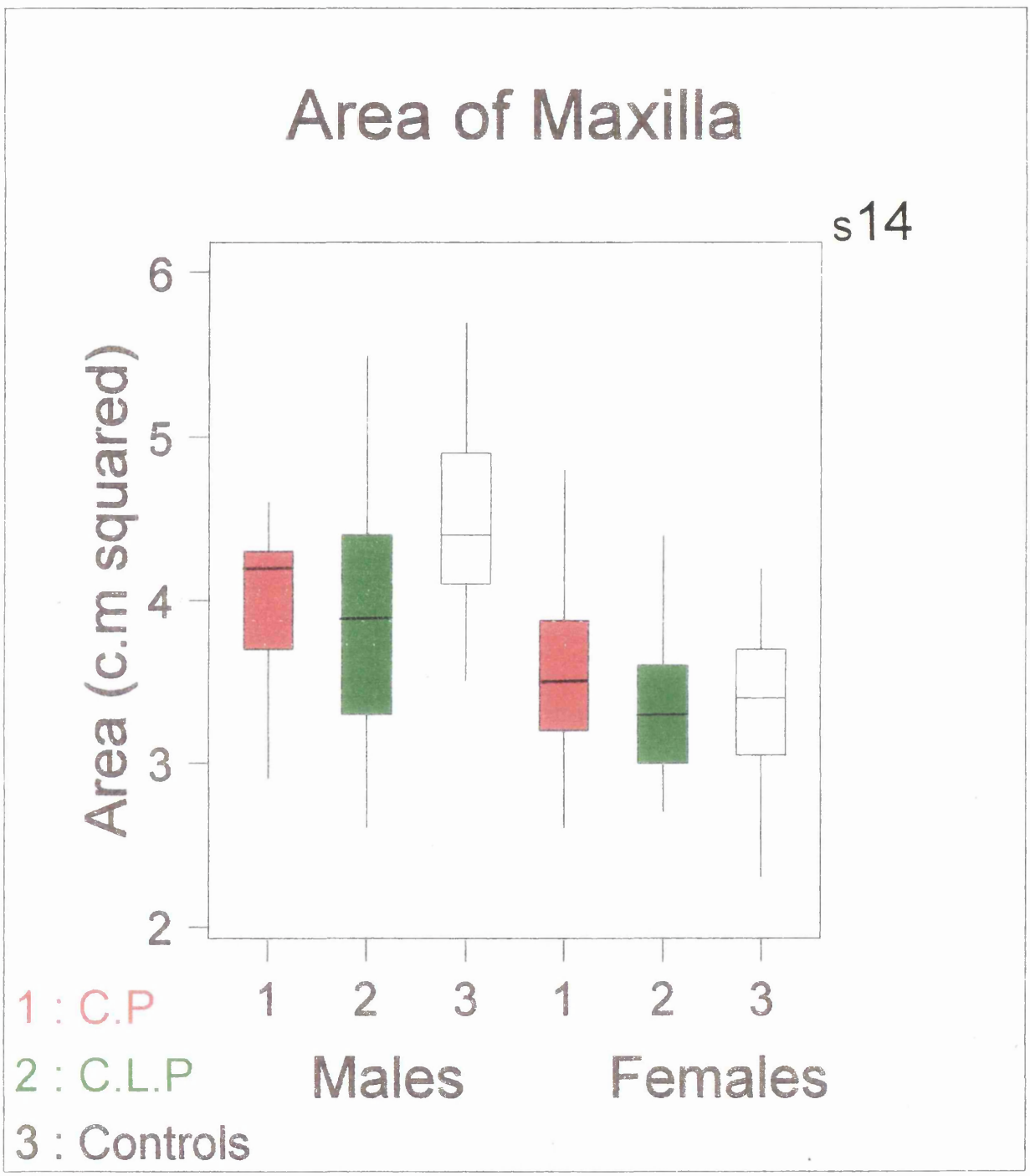

Area of Mandible
s2
Area (c.m squared)
40
30
20
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

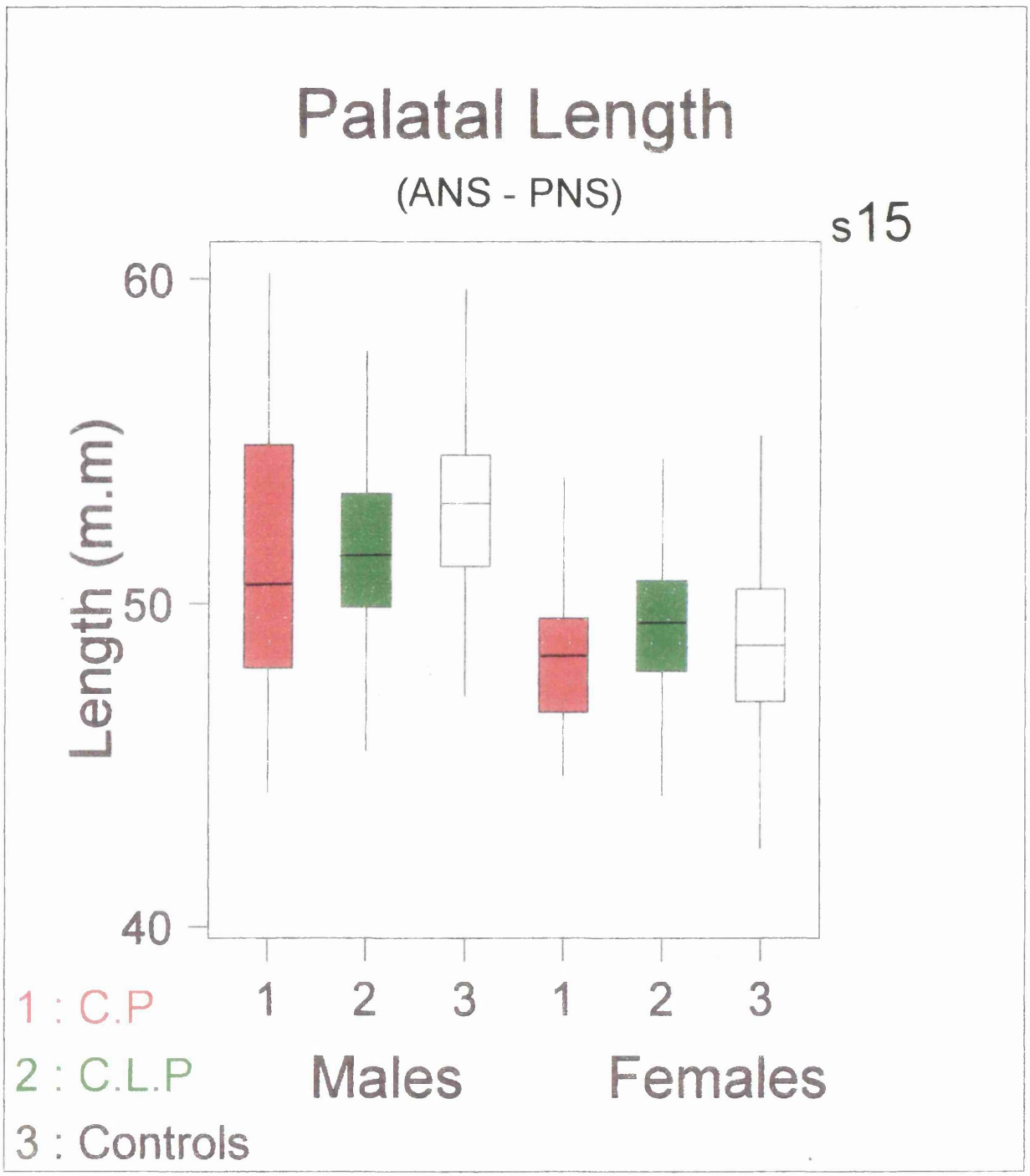

Body Length
(Rtan - Pog)
s3
Length (m.m)
90
80
70
60
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

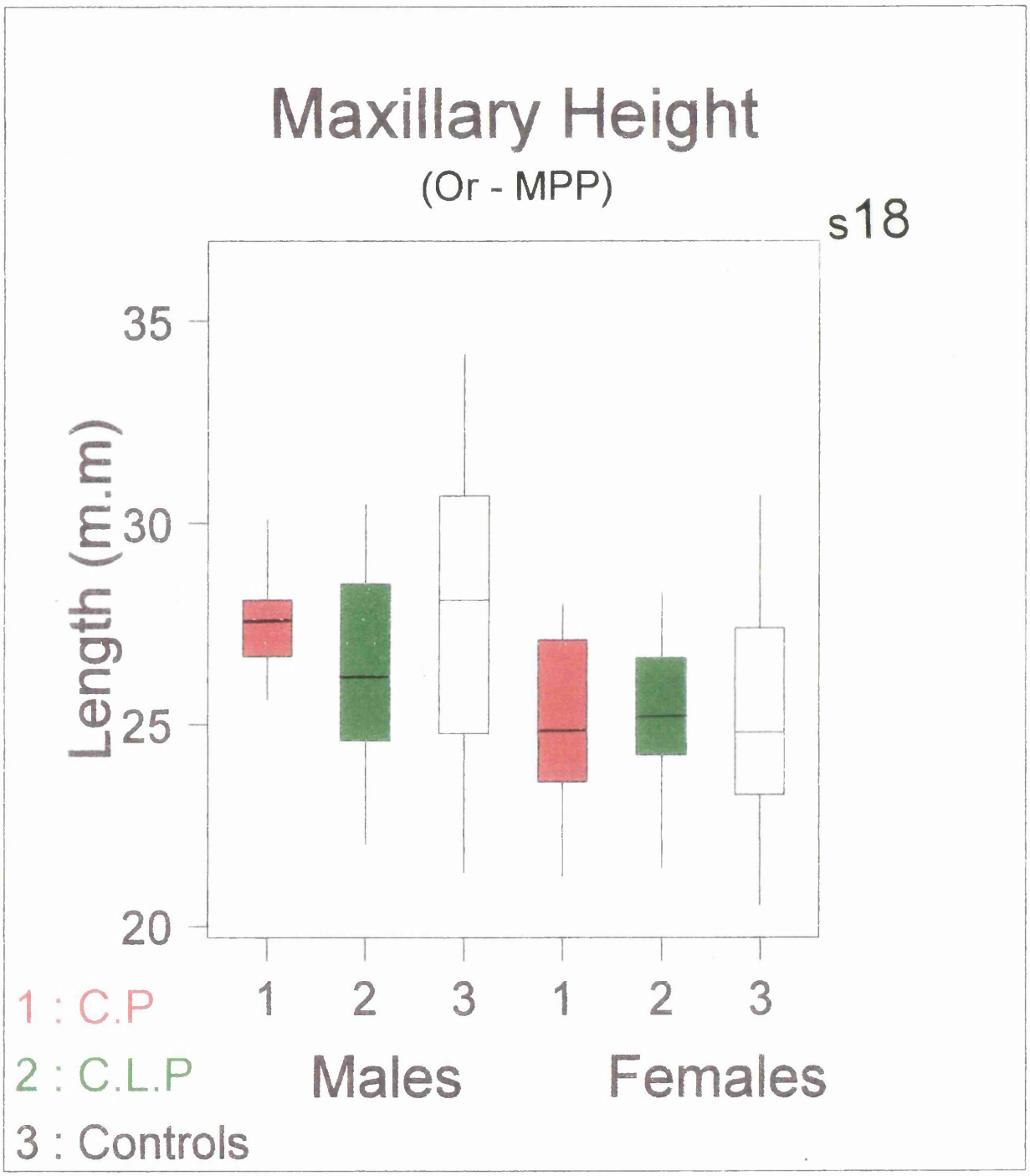

Mandibular Length
(Cd - Gn)
s7
125
115
105
95
Length (m.m)
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

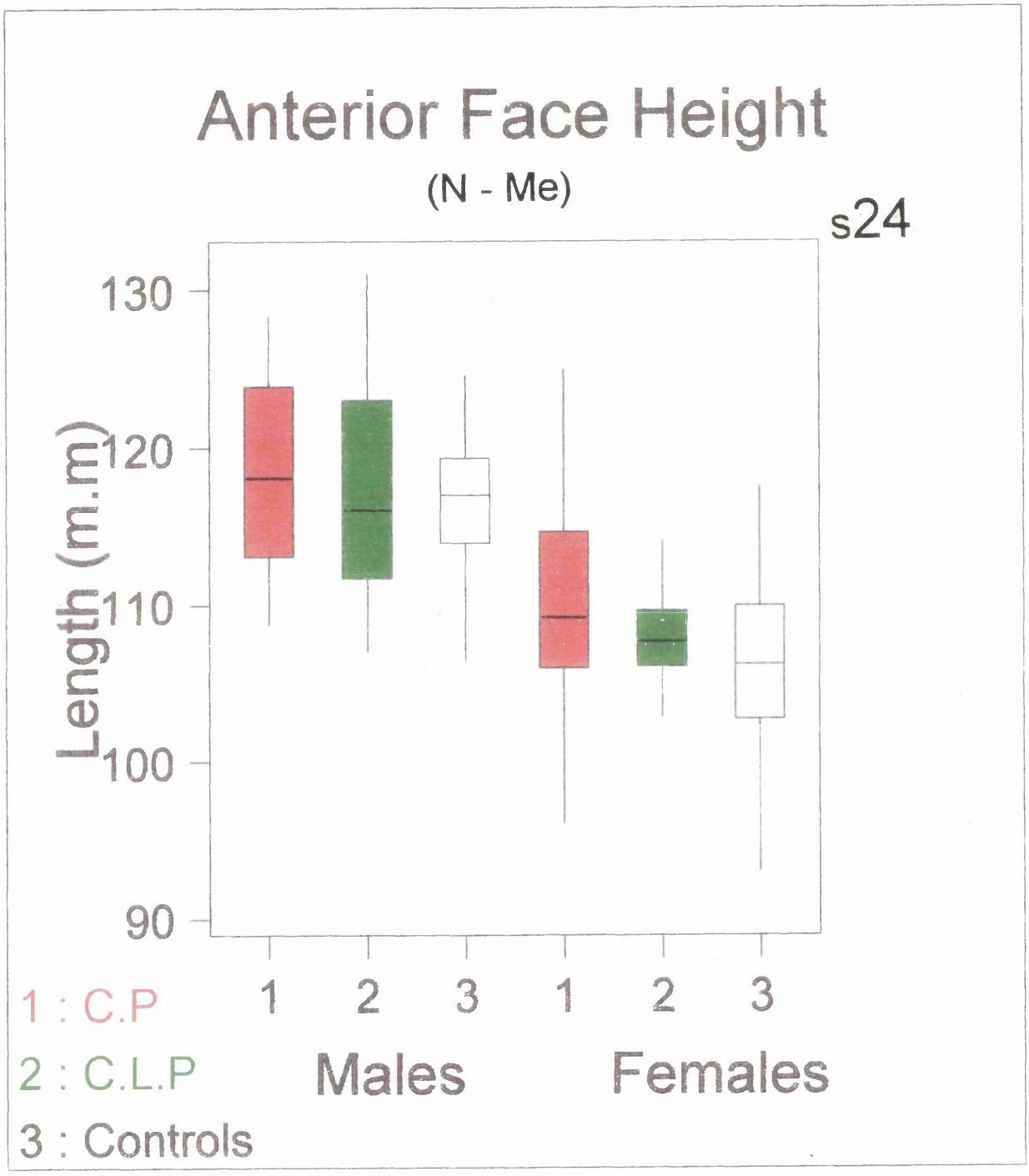

Ramus Length
(Cd - 14)
s8
80
70
60
50
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

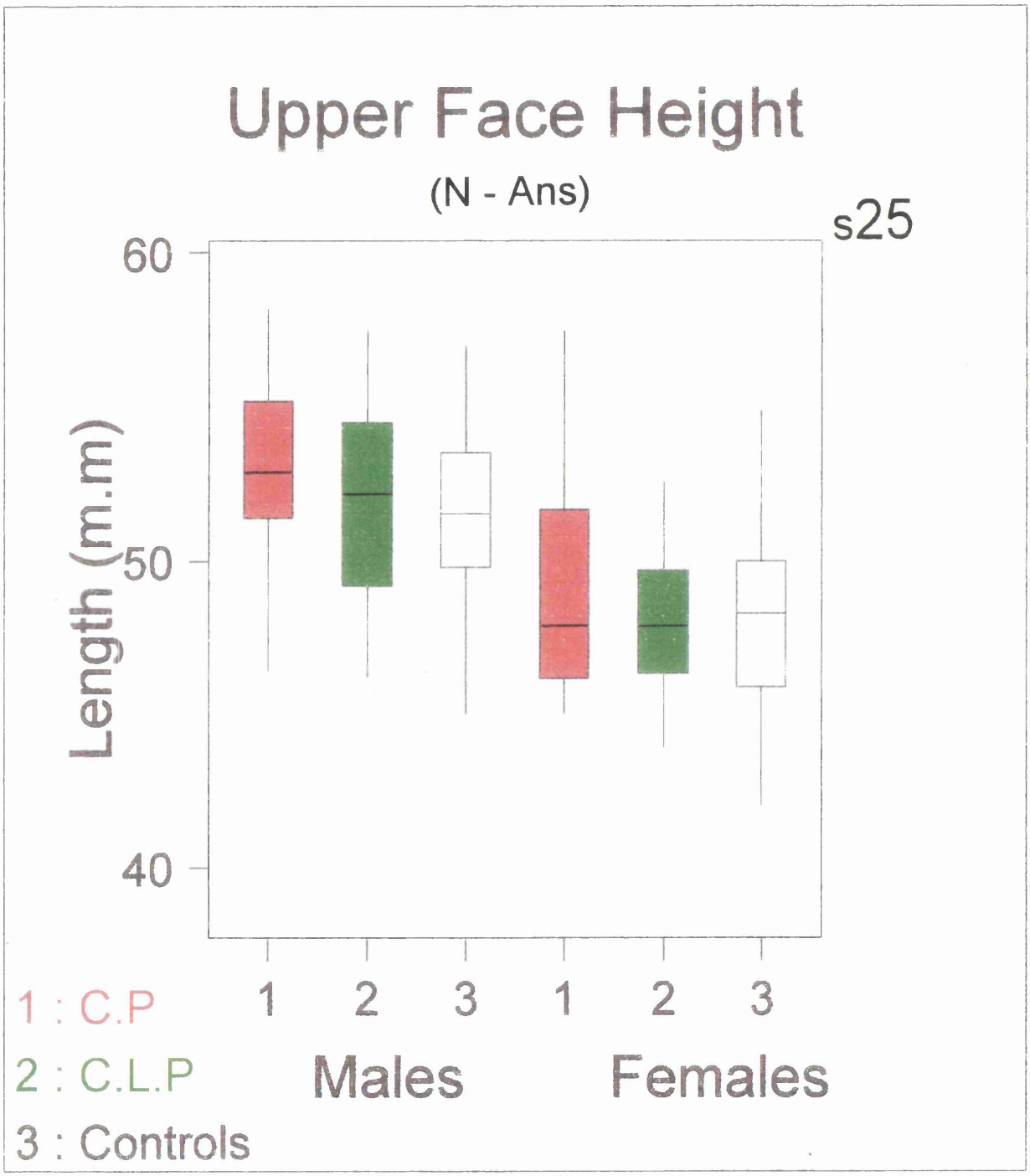

Gonial Angle
(Ar, Go, Me)
s11
145
135
125
115
105
Angle (degrees)
1
2
3
1
2
3
1 : C.P
2 : C.L.P
3 : Controls
Males
Females

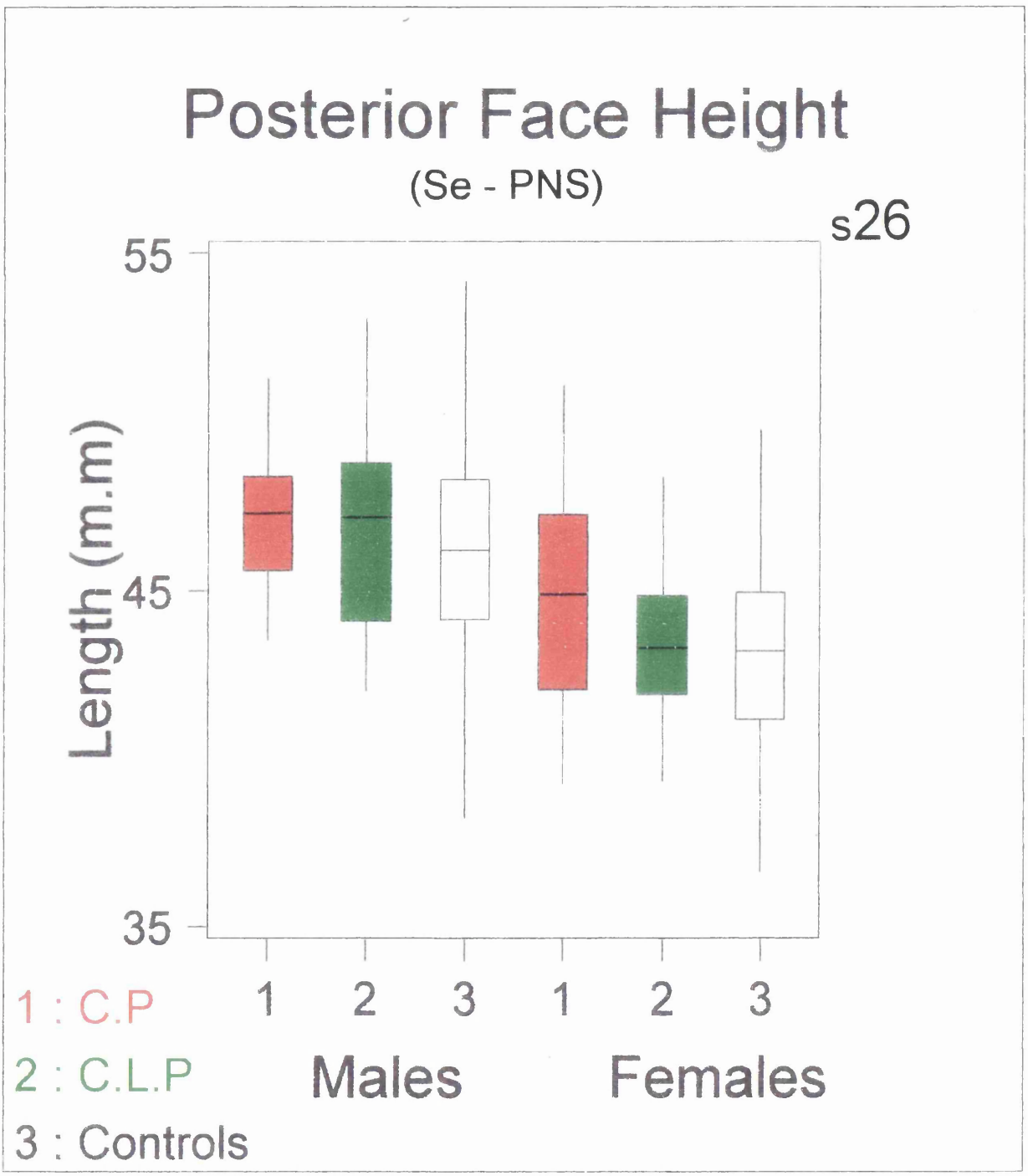

Area of Maxilla
s14
Area (c.m squared)
6
5
4
3
2
1
2
3
1
2
3
1 : C.P
2 : C.L.P
3 : Controls
Males
Females

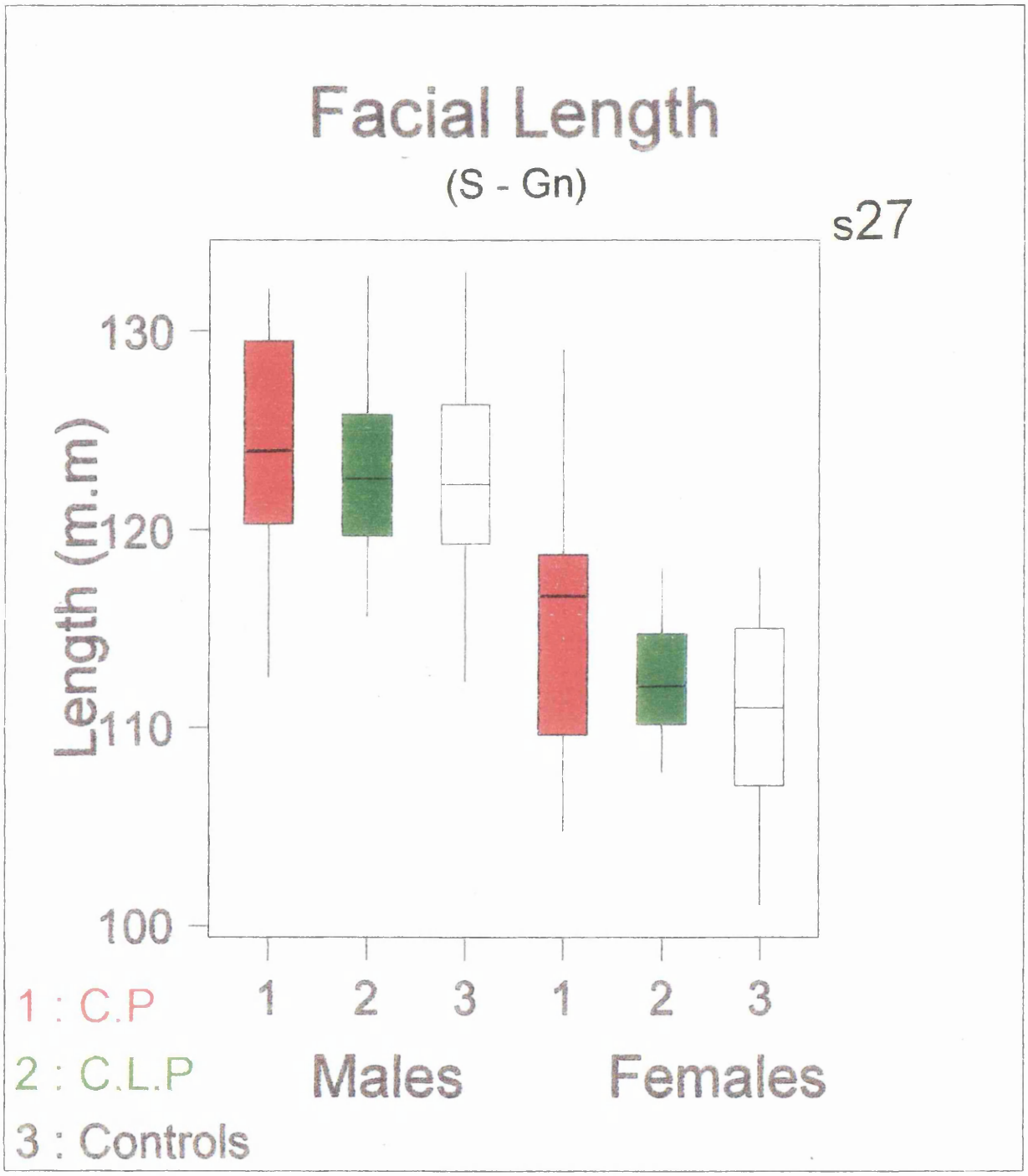

Palatal Length
(ANS - PNS)
s15
60
50
40
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

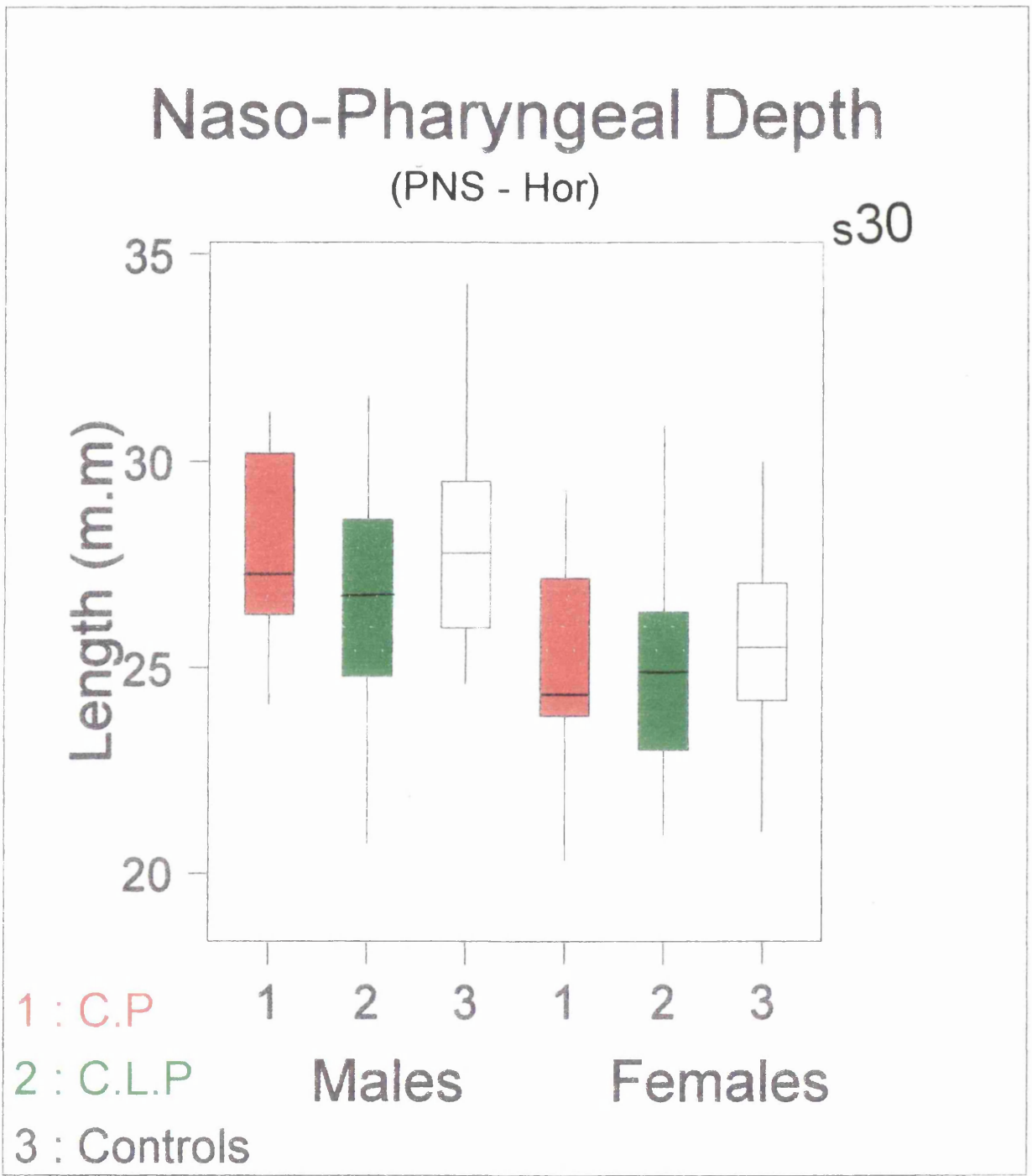

Maxillary Height
(Or - MPP)
s18
Length (m.m)
35
30
25
20
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

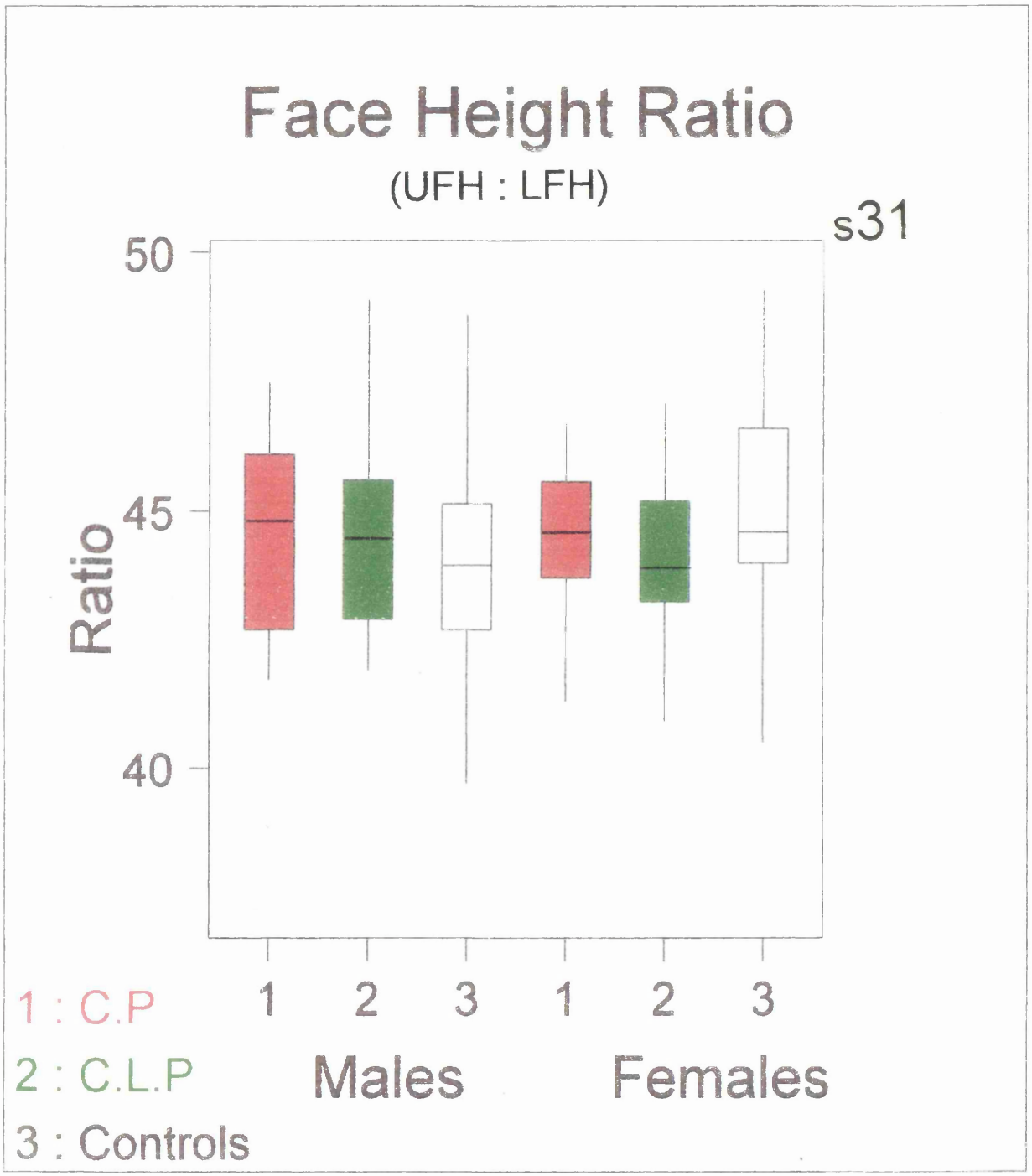

Anterior Face Height
(N - Me)
s24
Length (m.m)
130
120
110
100
90
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

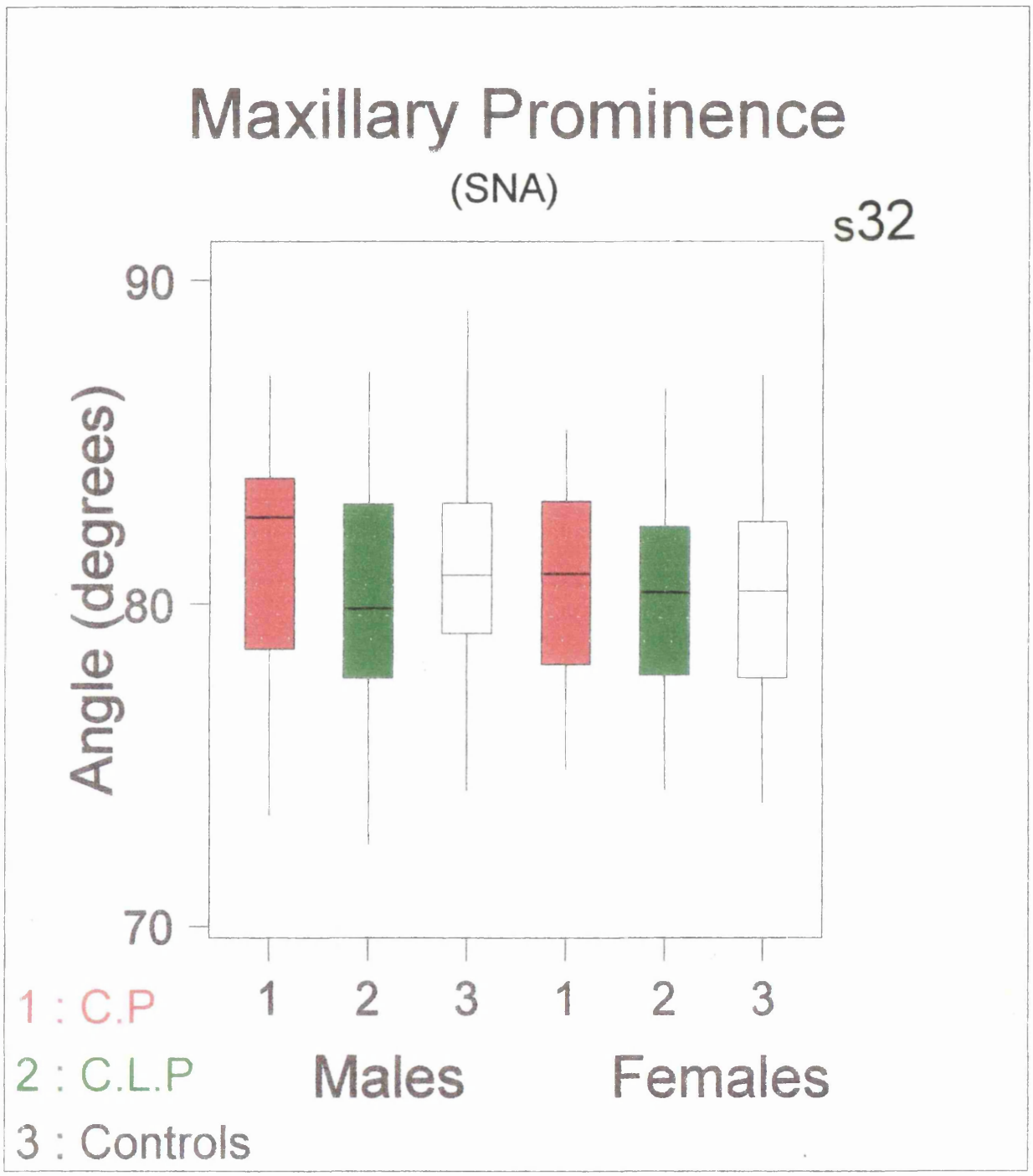

Upper Face Height
(N - Ans)
s25
60
50
40
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

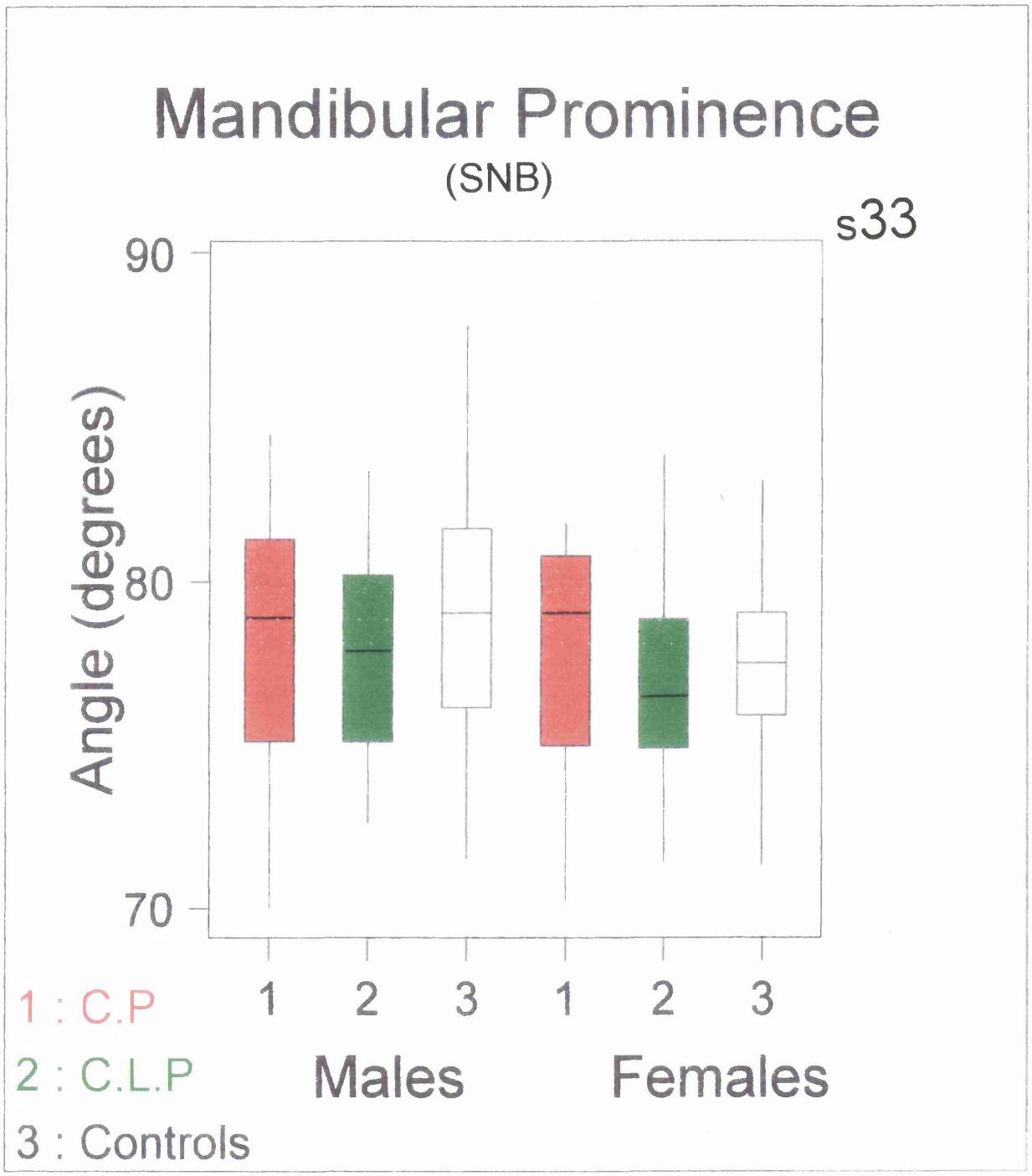

Posterior Face Height
(Se - PNS)
s26
55
45
35
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

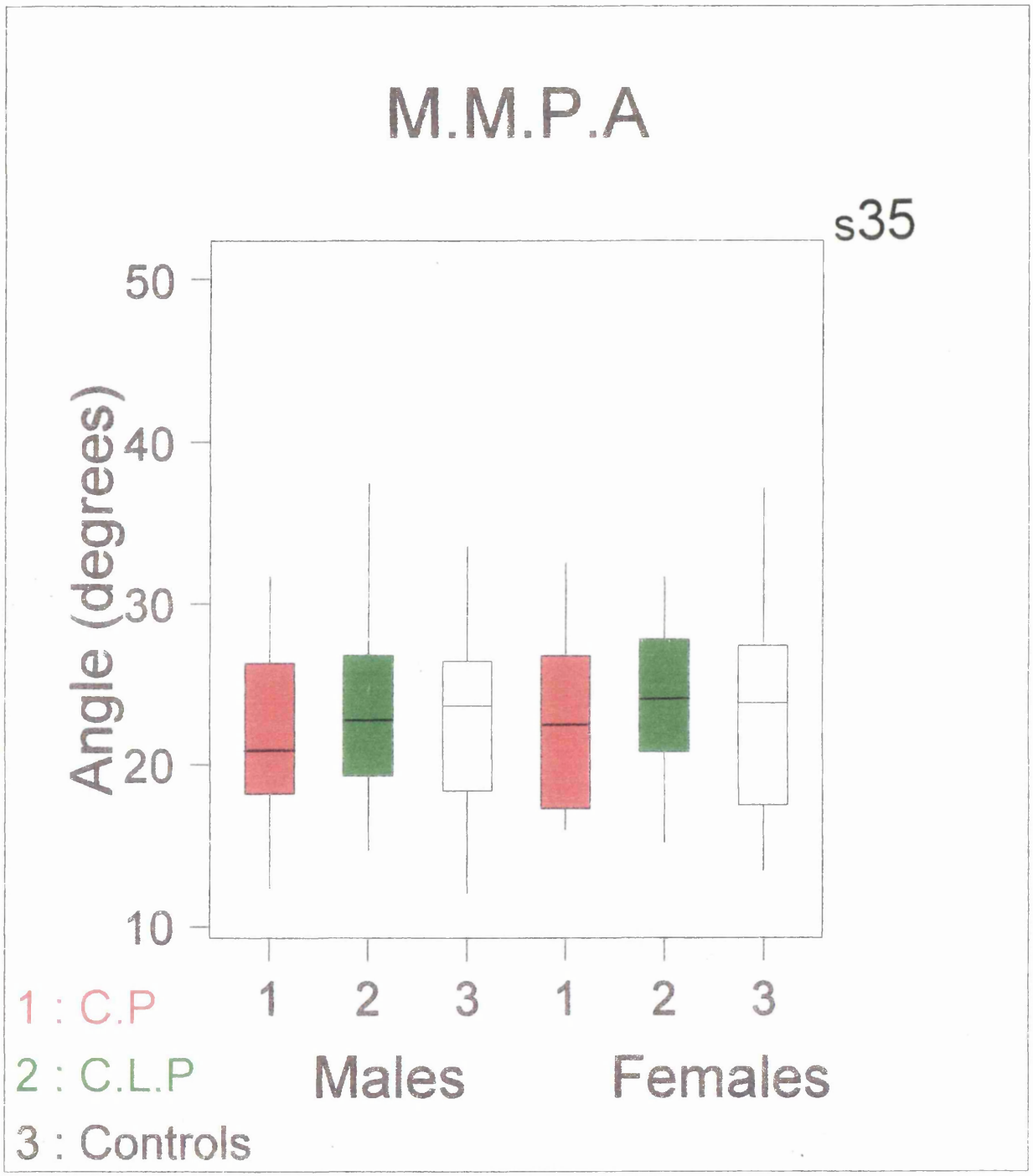

Facial Length
(S - Gn)
s27
Length (m.m)
130
120
110
100
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

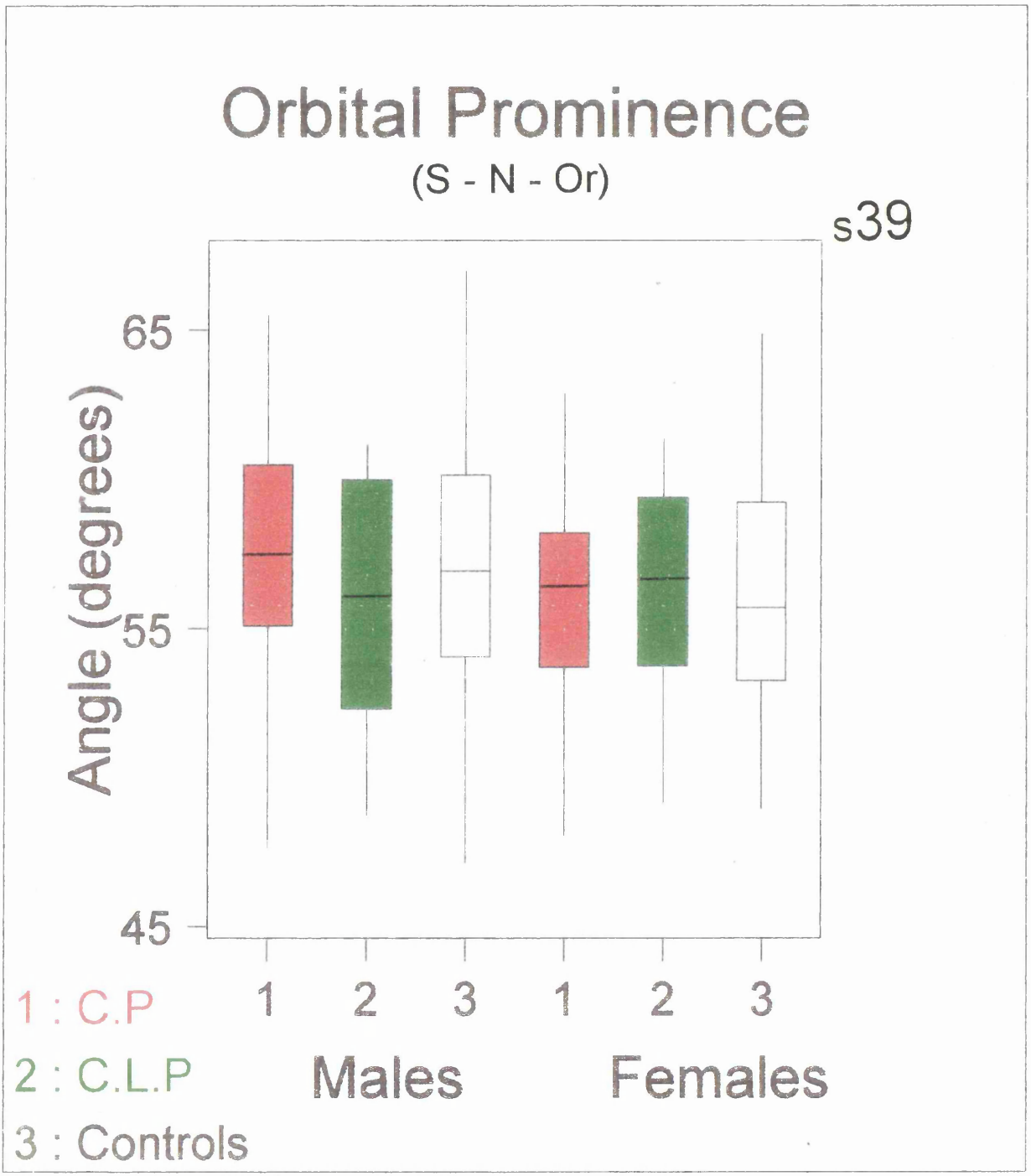

Naso-Pharyngeal Depth
(PNS - Hor)
s30
Length (m.m)
35
30
25
20
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

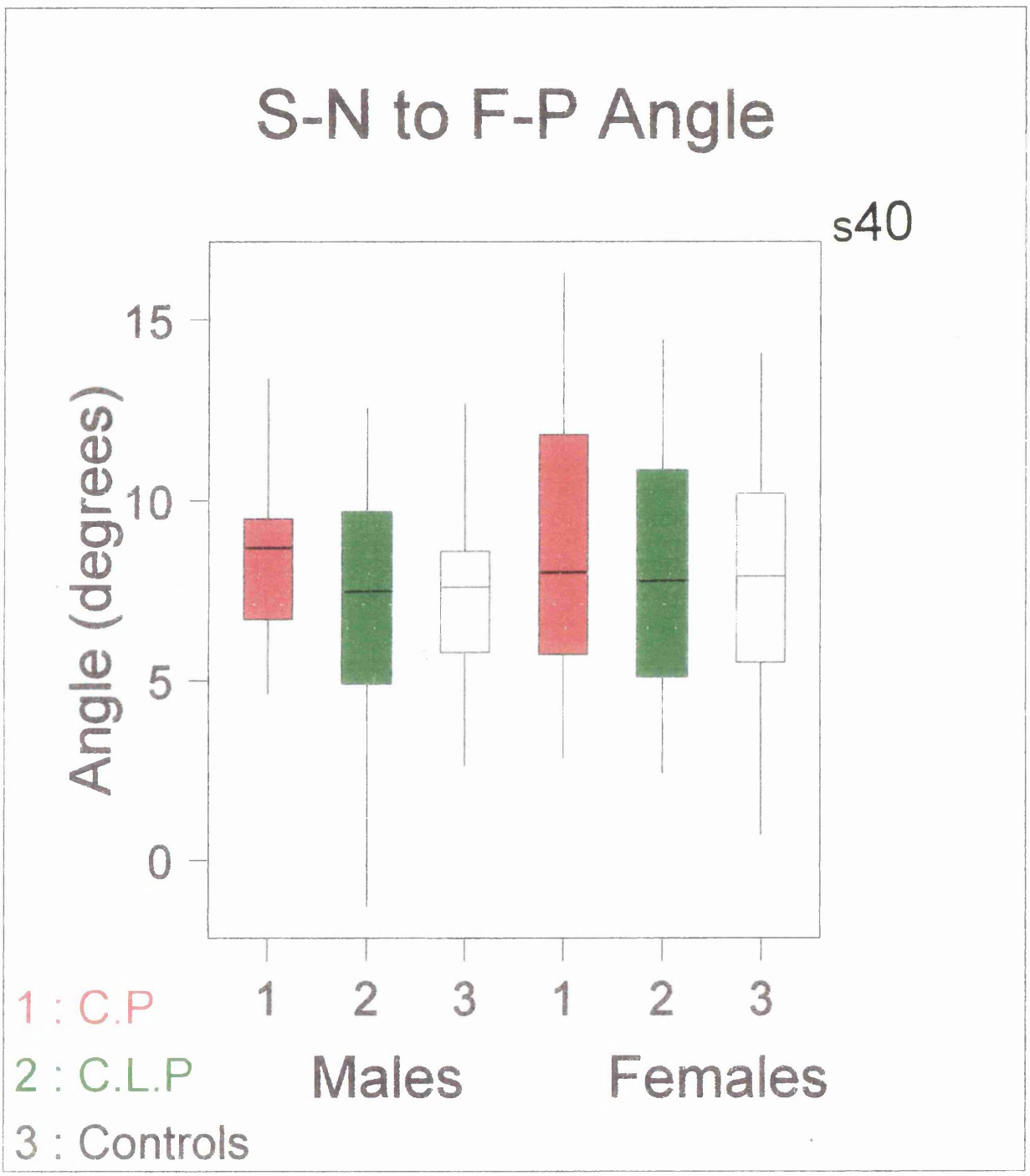

Face Height Ratio
(UFH : LFH)
s31
50
45
40
Ratio
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

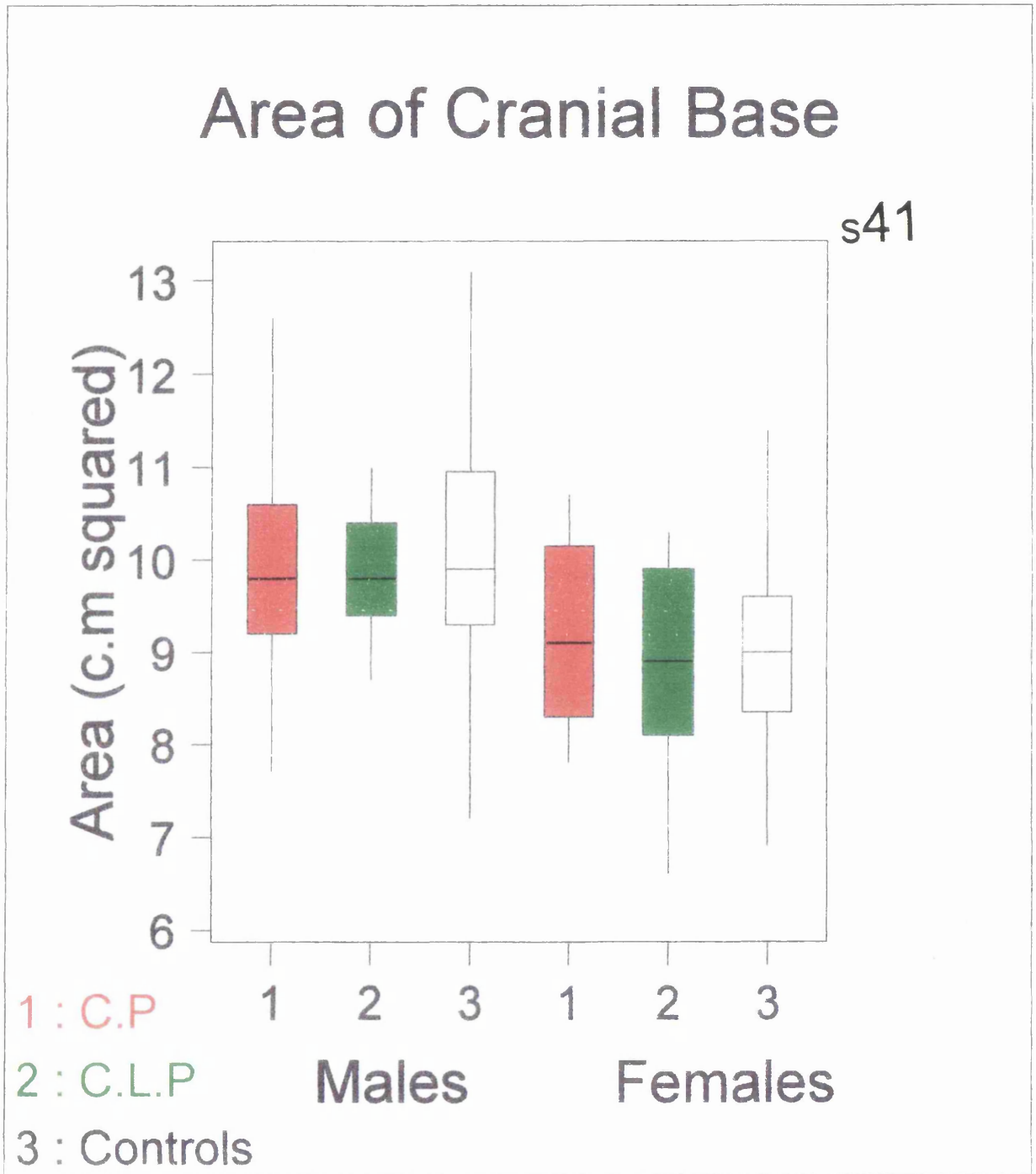

Maxillary Prominence
(SNA)
s32
90
80
70
Angle (degrees)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

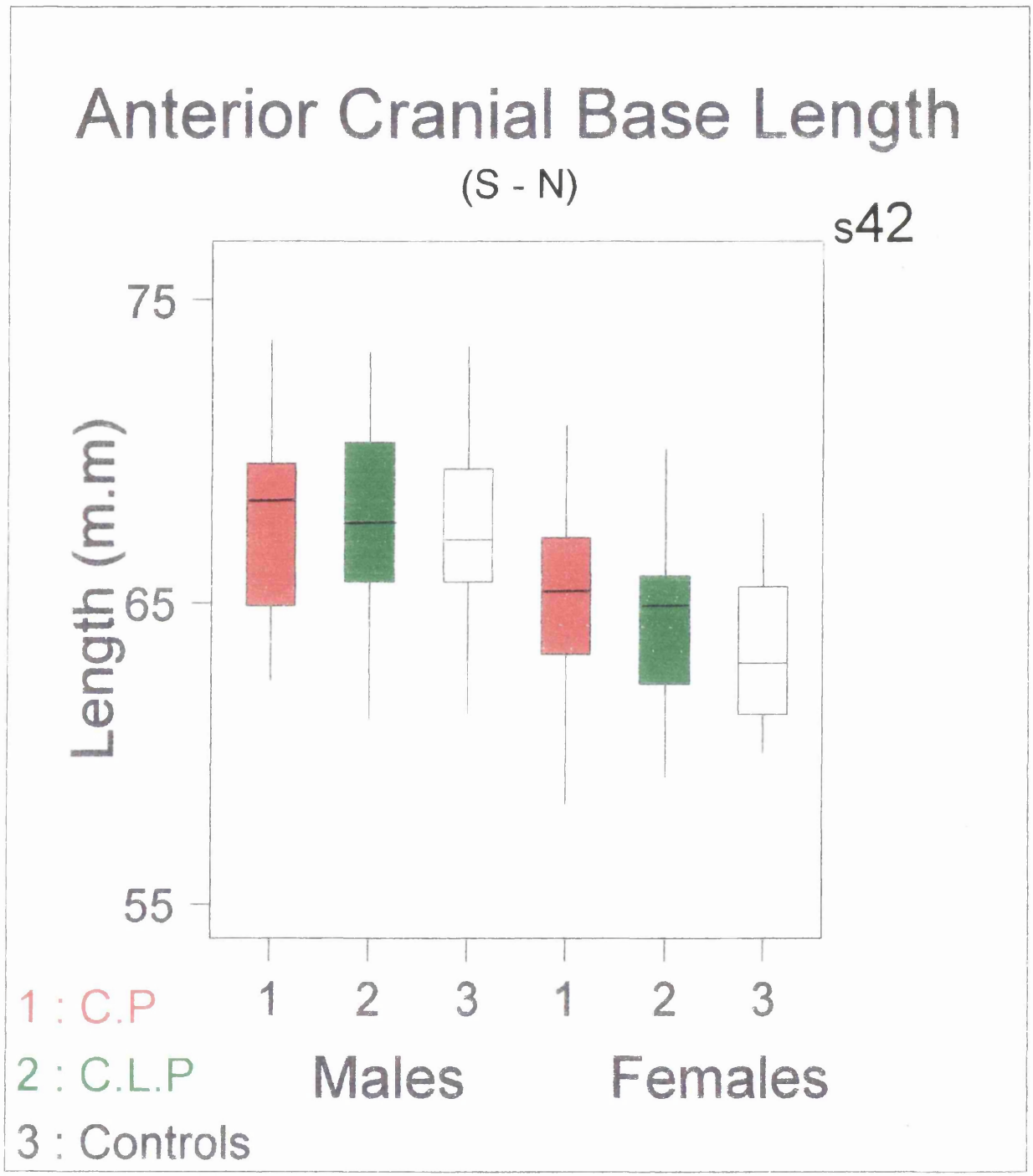

Mandibular Prominence
(SNB)
s33
90
80
70
Angle (degrees)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

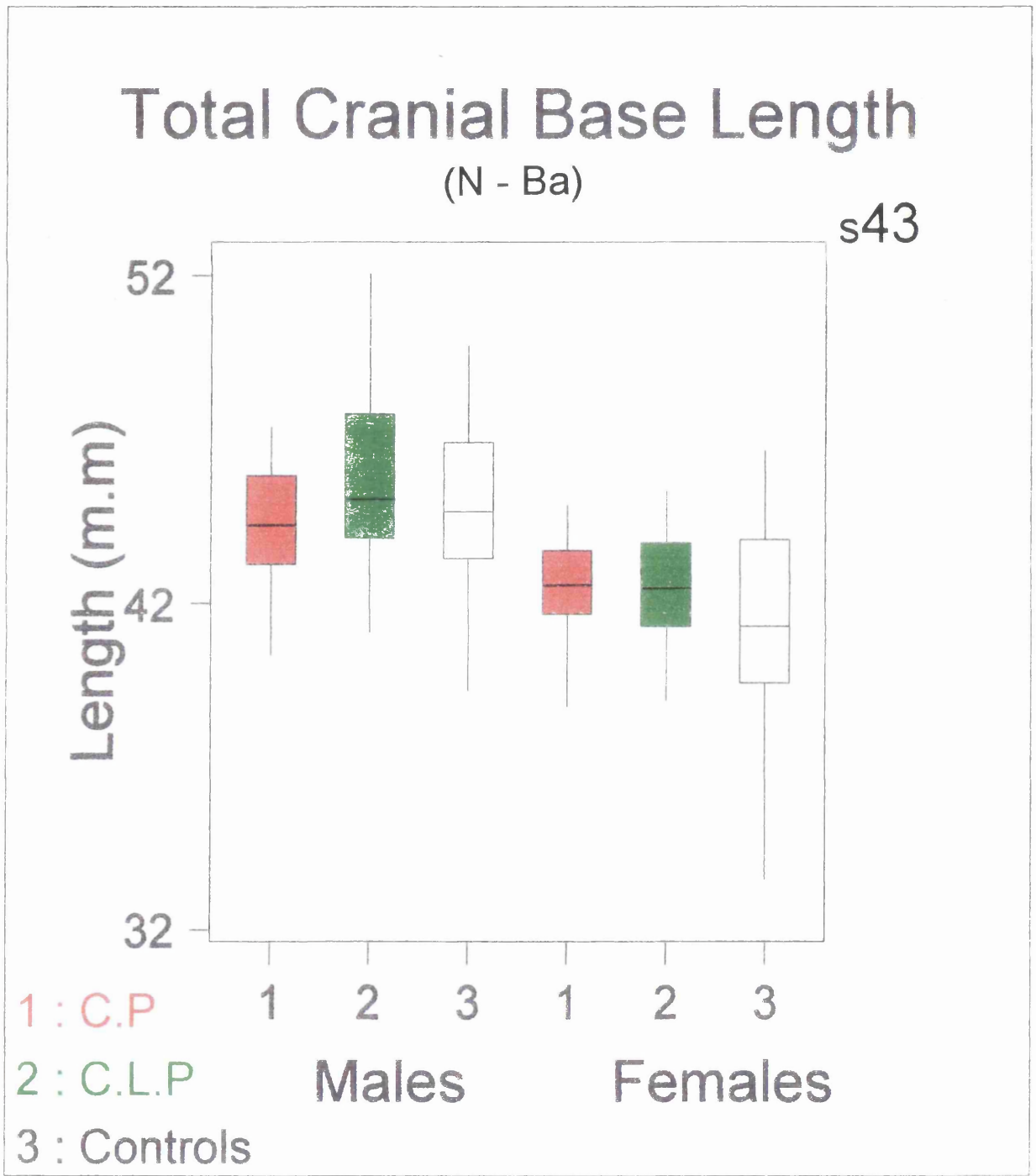

M.M.P.A
s35
Angle (degrees)
50
40
30
20
10
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

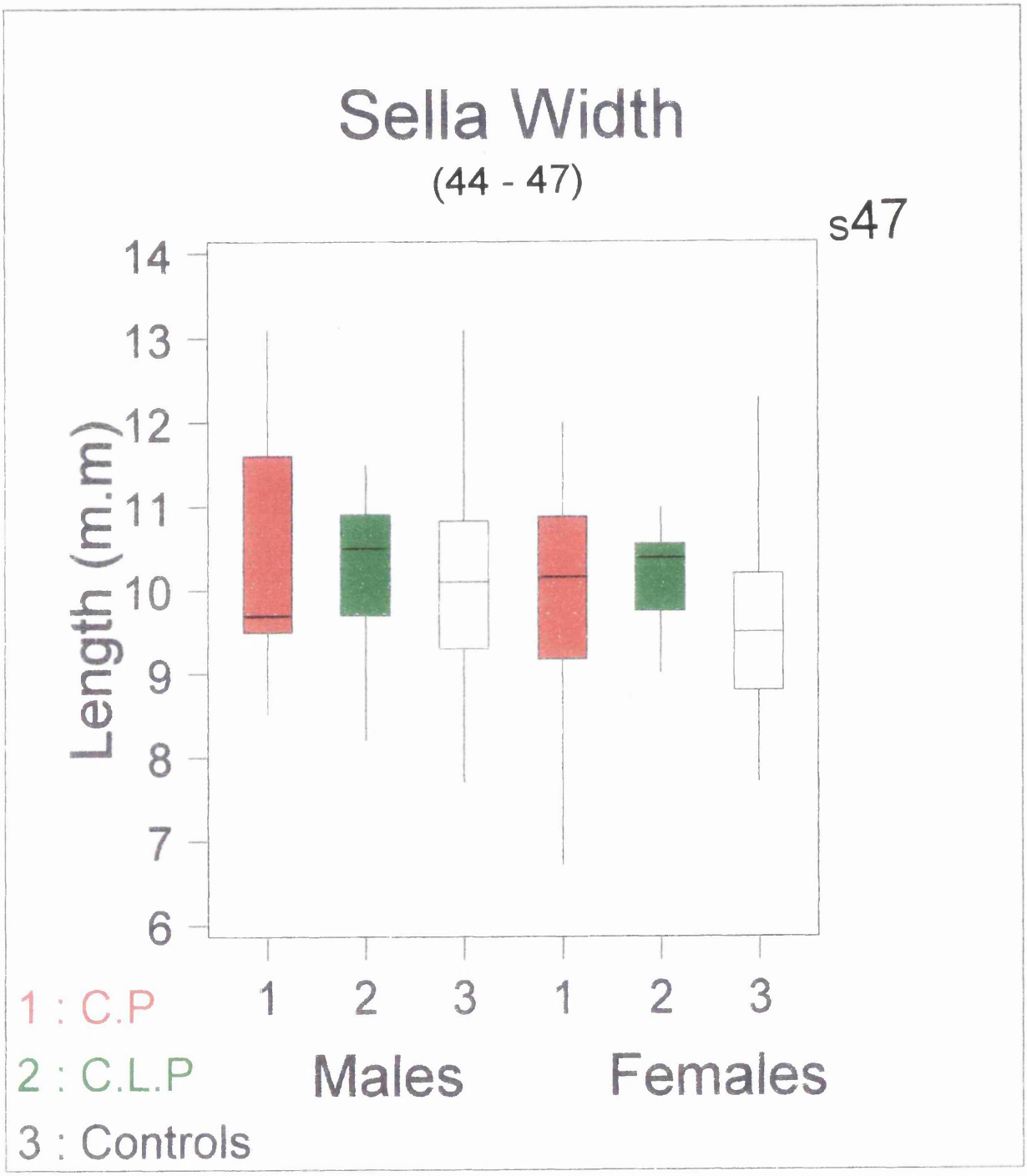

Orbital Prominence
(S - N - Or)
s39
65
55
45
Angle (degrees)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

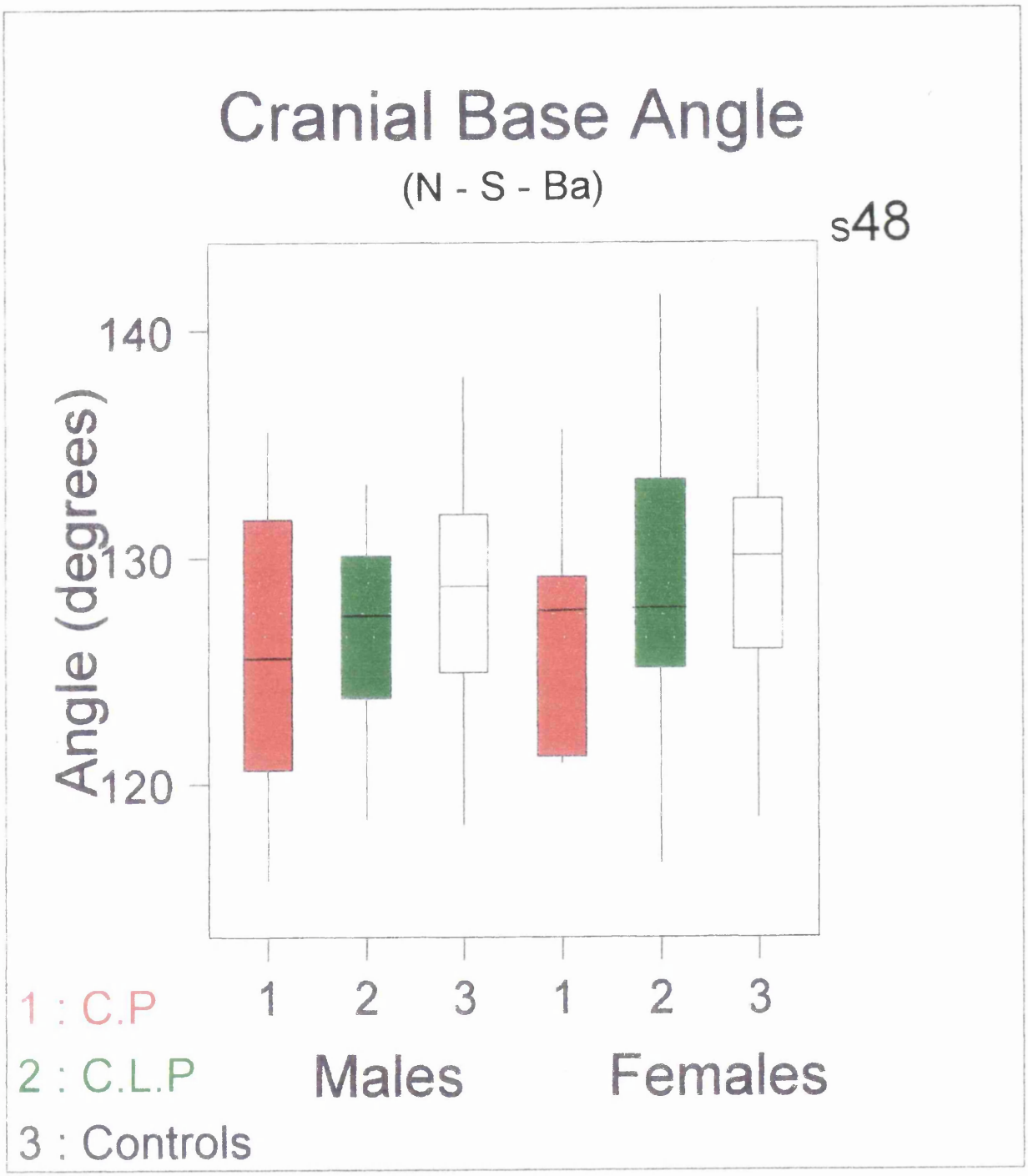

S-N to F-P Angle
s40
Angle (degrees)
15
10
5
0
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

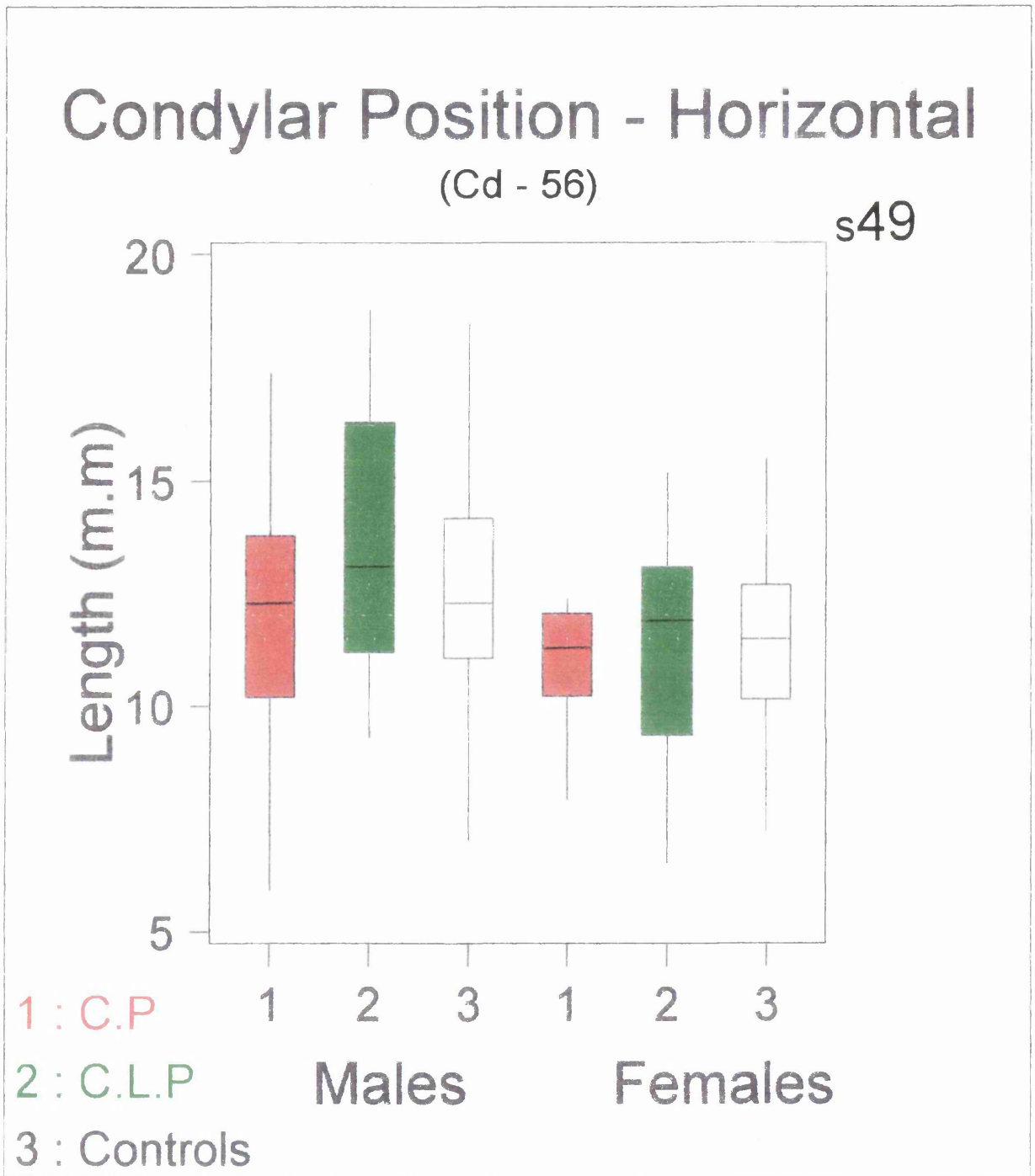

Area of Cranial Base
s41
Area (c.m squared)
13
12
11
10
9
8
7
6
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

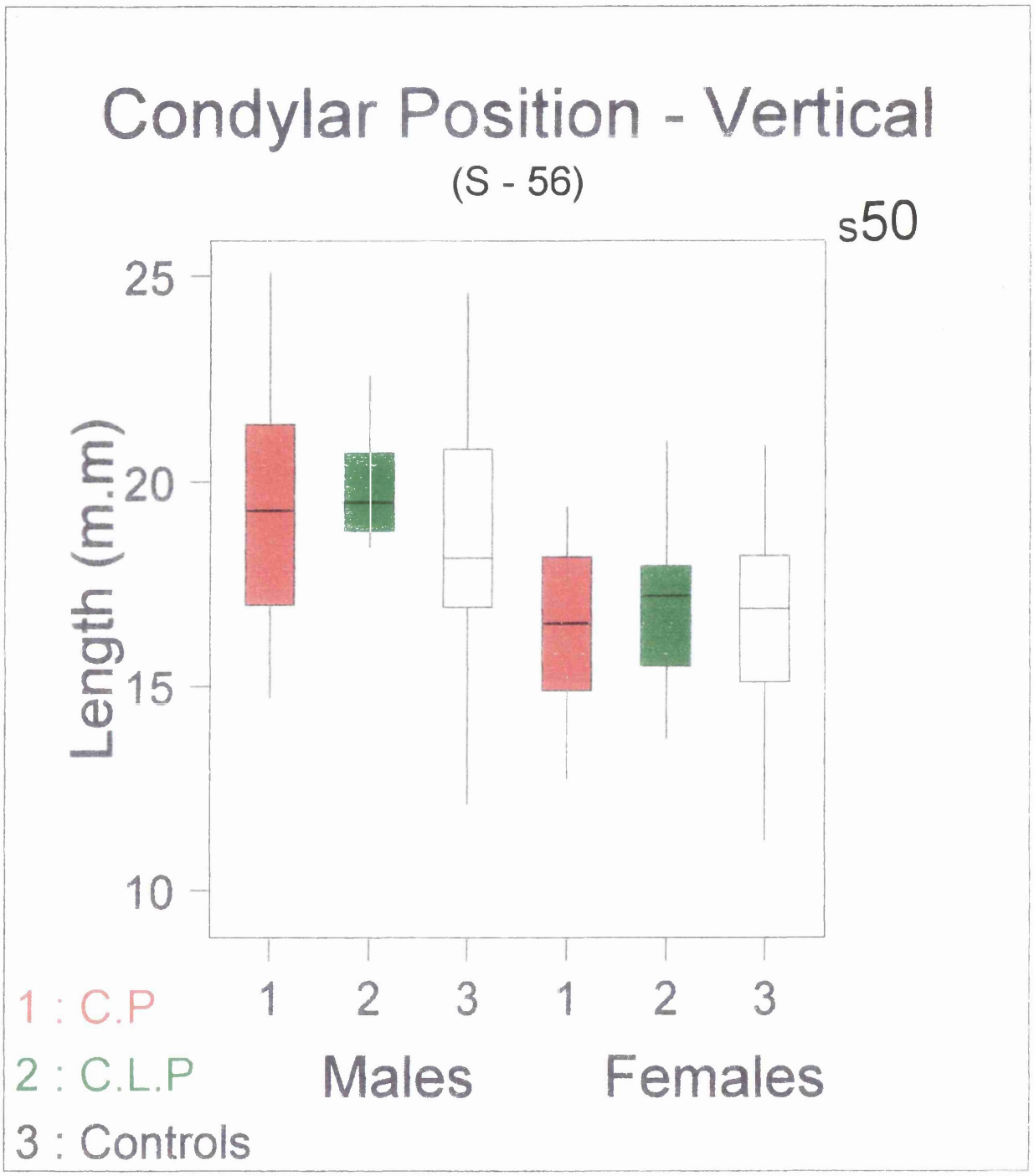

Anterior Cranial Base Length
(S - N)
s42
75
65
55
Length (m.m)
1
2
3
1
2
3
1 : C.P
2 : C.L.P
3 : Controls
Males
Females

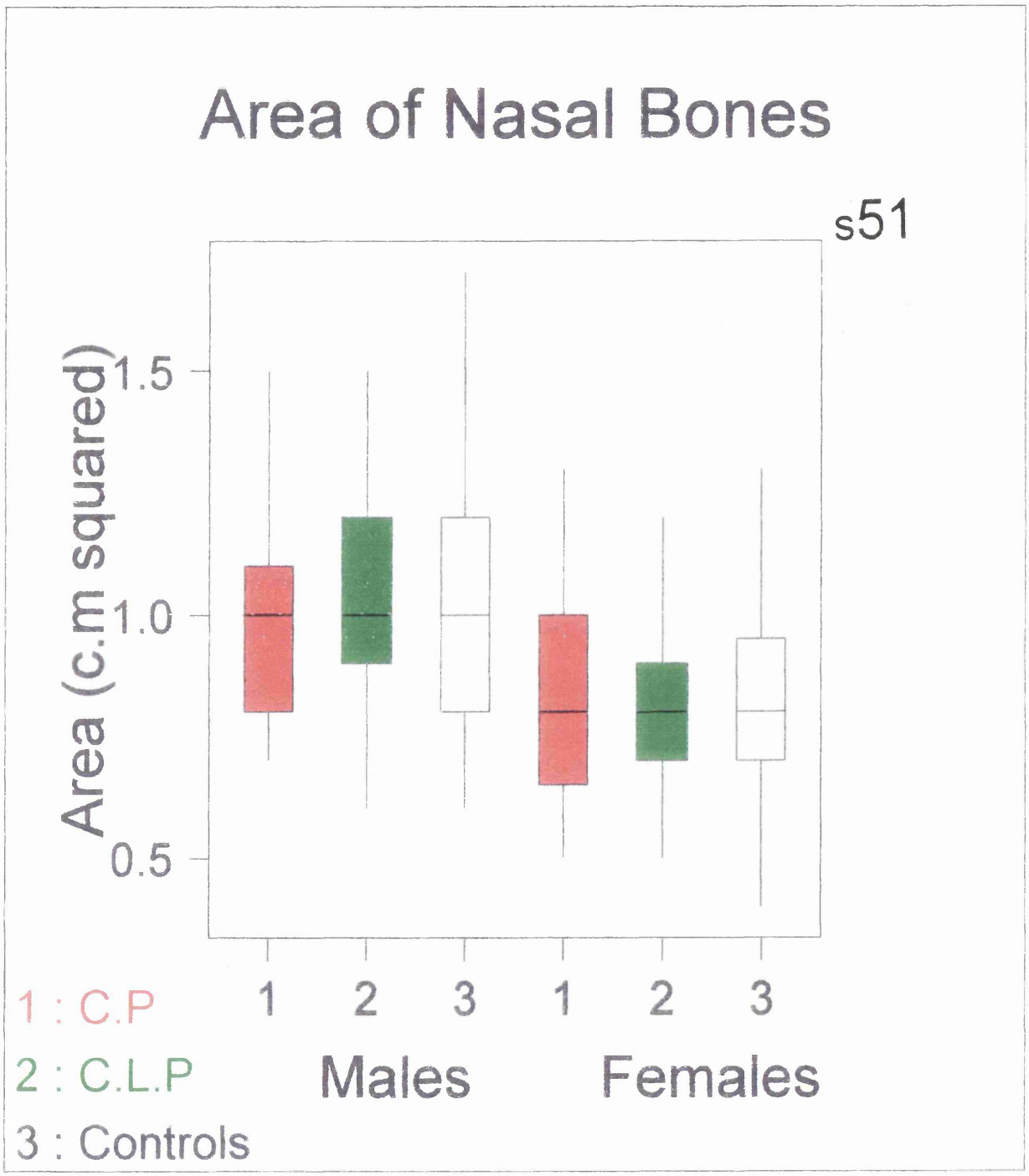

Total Cranial Base Length
(N - Ba)
s43
52
42
32
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

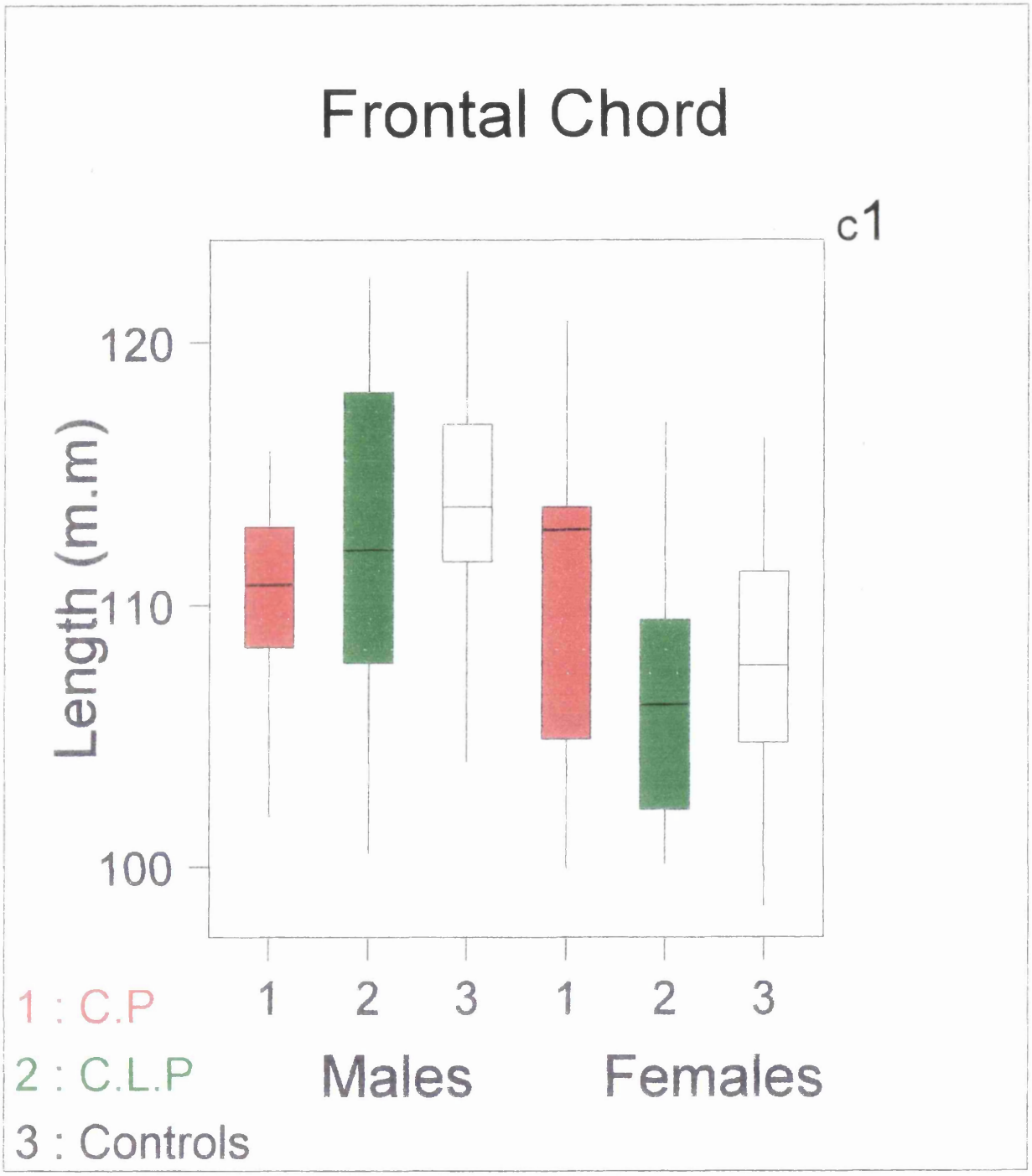

Sella Width
(44 - 47)
s47
Length (m.m)
14
13
12
11
10
9
8
7
6
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

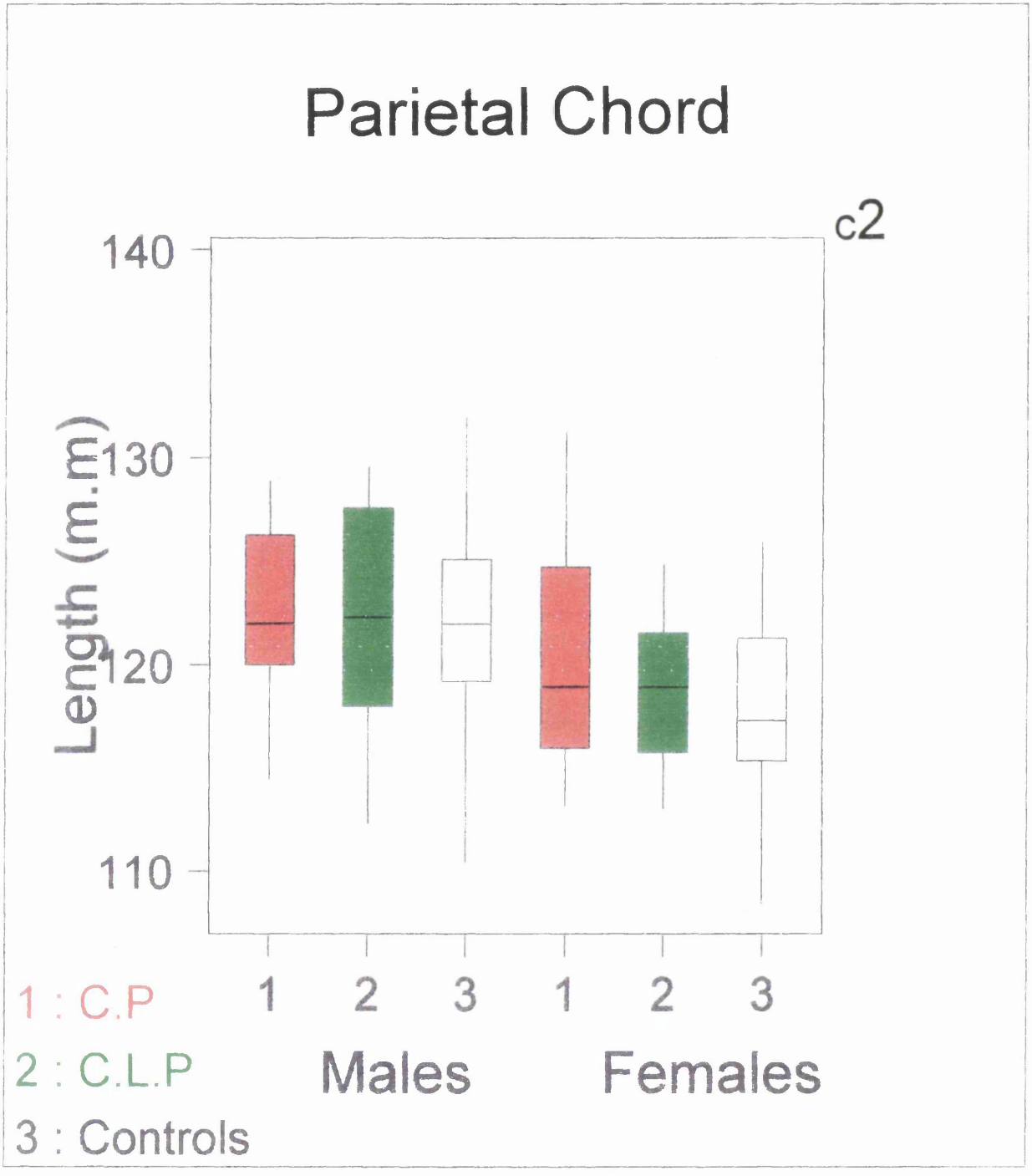

Cranial Base Angle
(N - S - Ba)
s48
Angle (degrees)
140
130
120
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

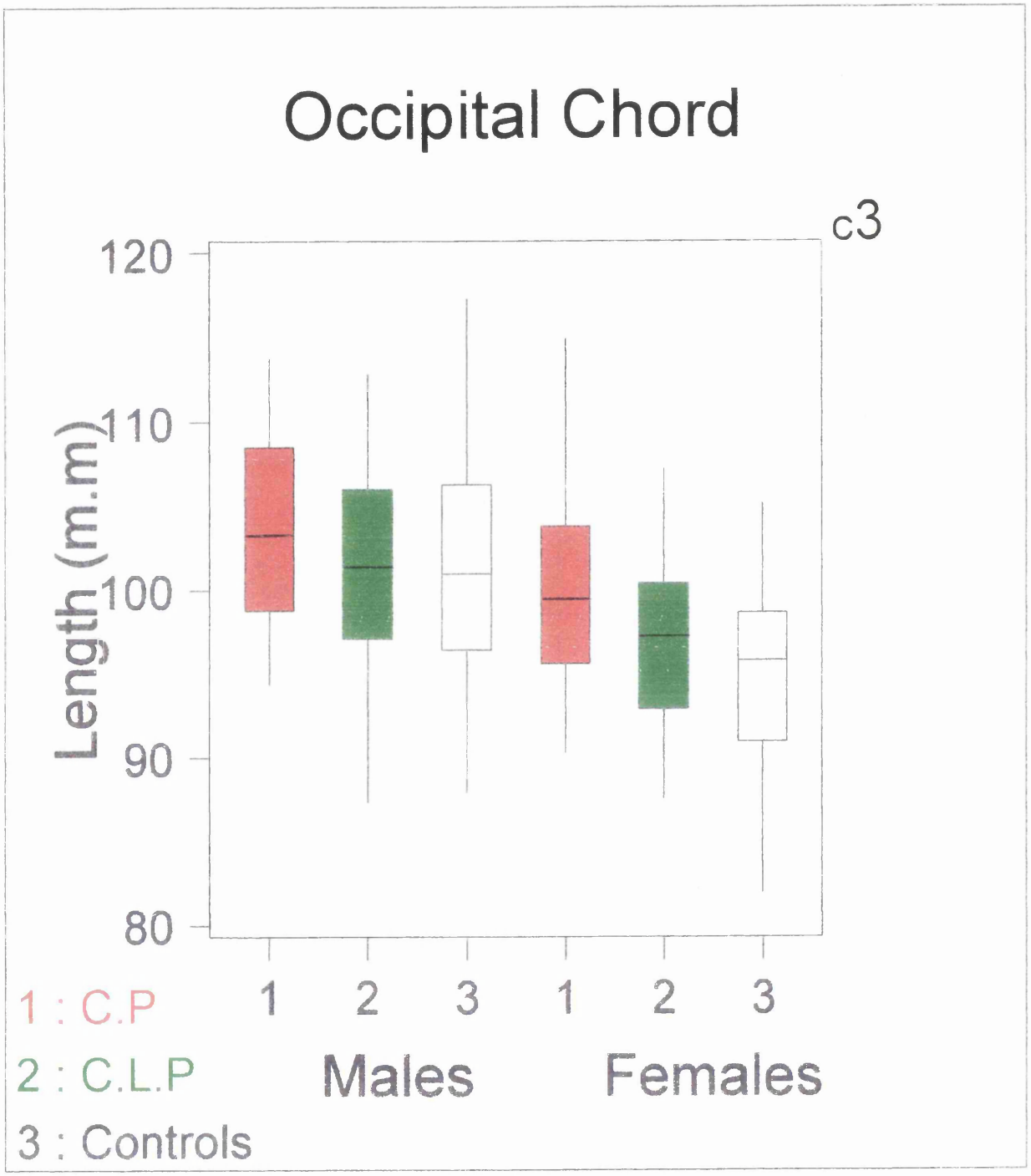

Condylar Position - Horizontal
(Cd - 56)
s49
Length (m.m)
20
15
10
5
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

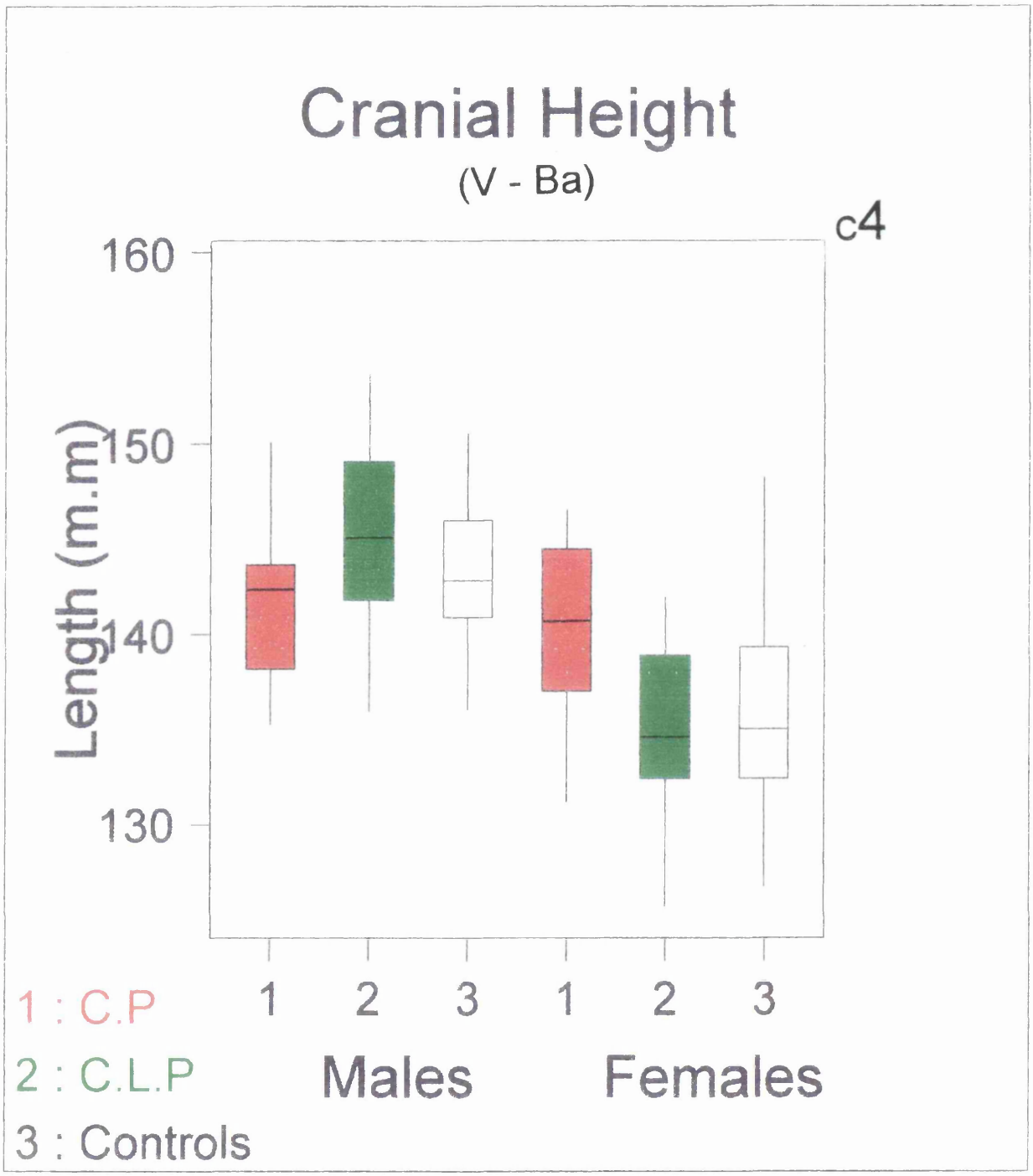

Condylar Position - Vertical
(S - 56)
s50
Length (m.m)
25
20
15
10
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

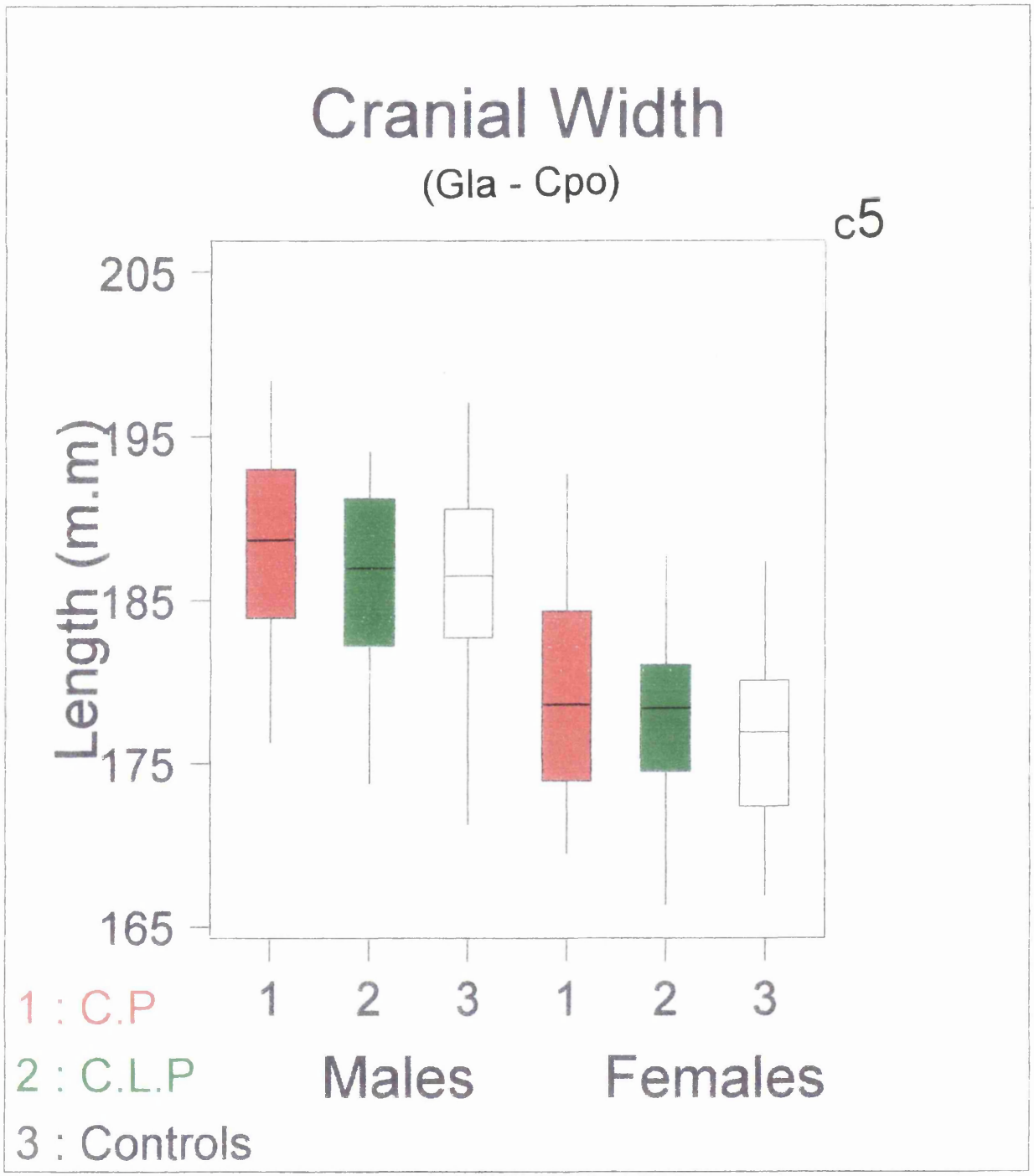

Area of Nasal Bones
s51
Area (c.m squared)
1.5
1.0
0.5
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

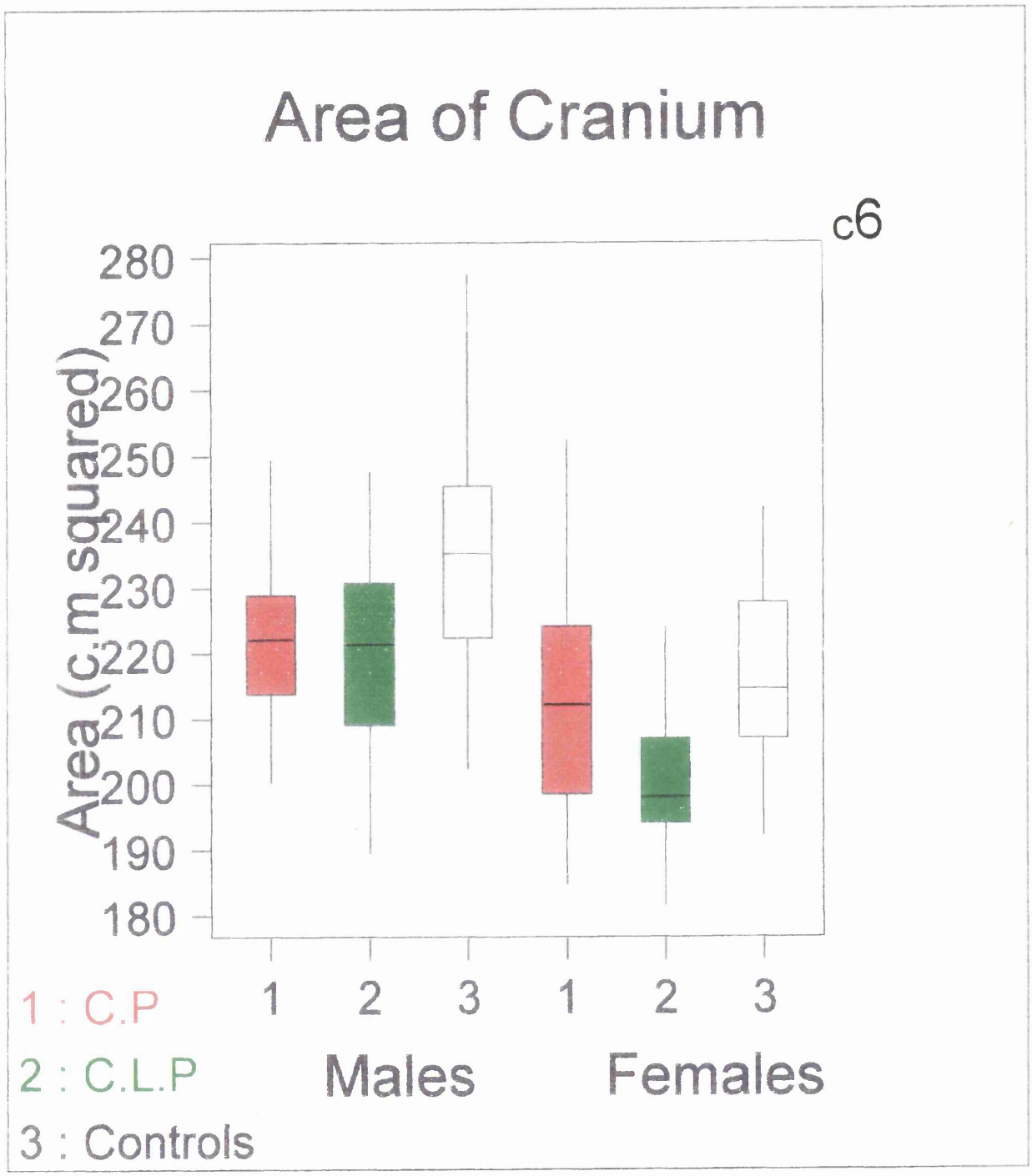

Frontal Chord
c1
Length (m.m)
120
110
100
1
2
3
1
2
3
1 : C.P
2 : C.L.P
3 : Controls
Males
Females

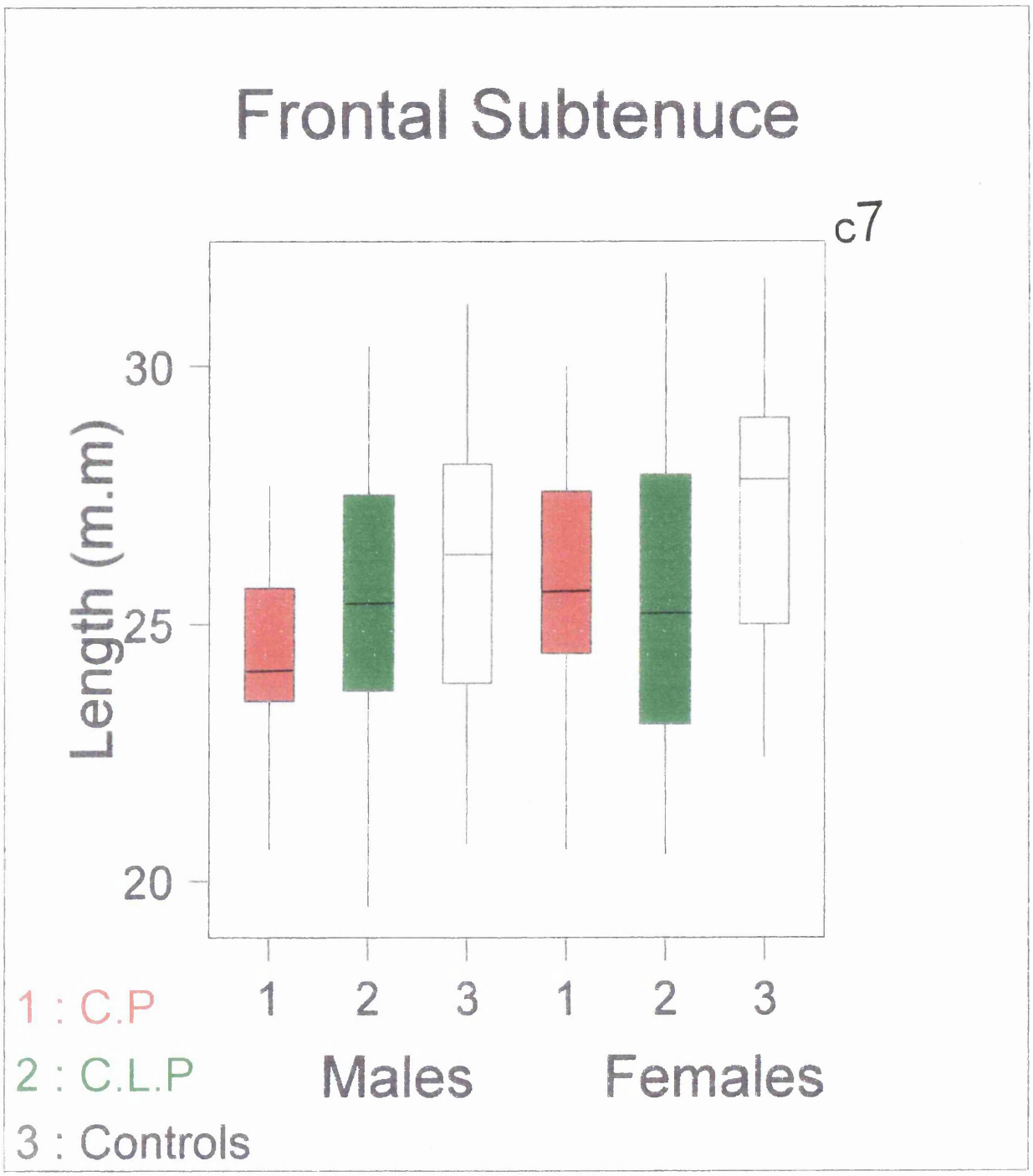

Parietal Chord
c2
140
130
120
110
Length (m.m)
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls

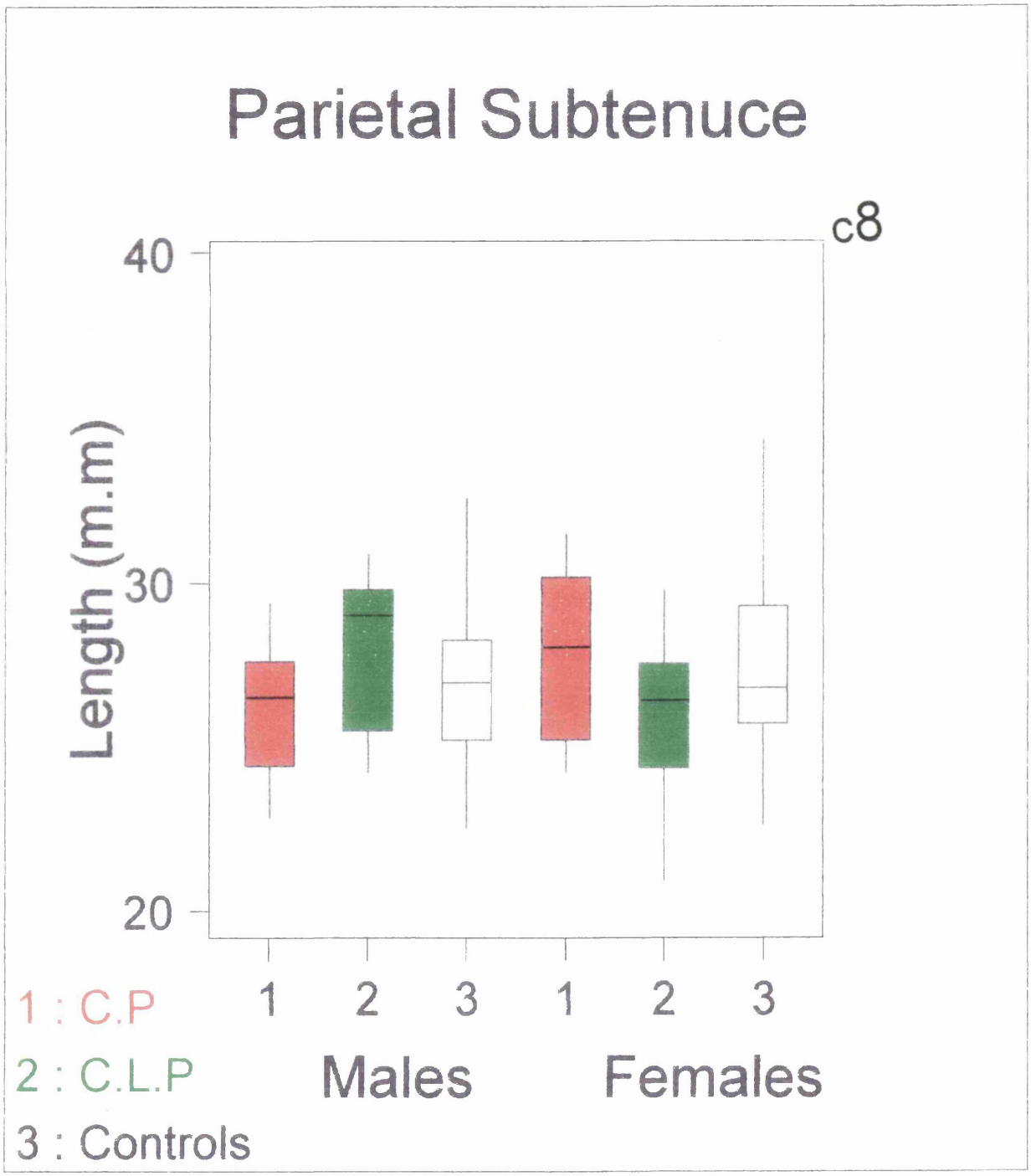

Occipital Chord
c3
120
110
100
90
80
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

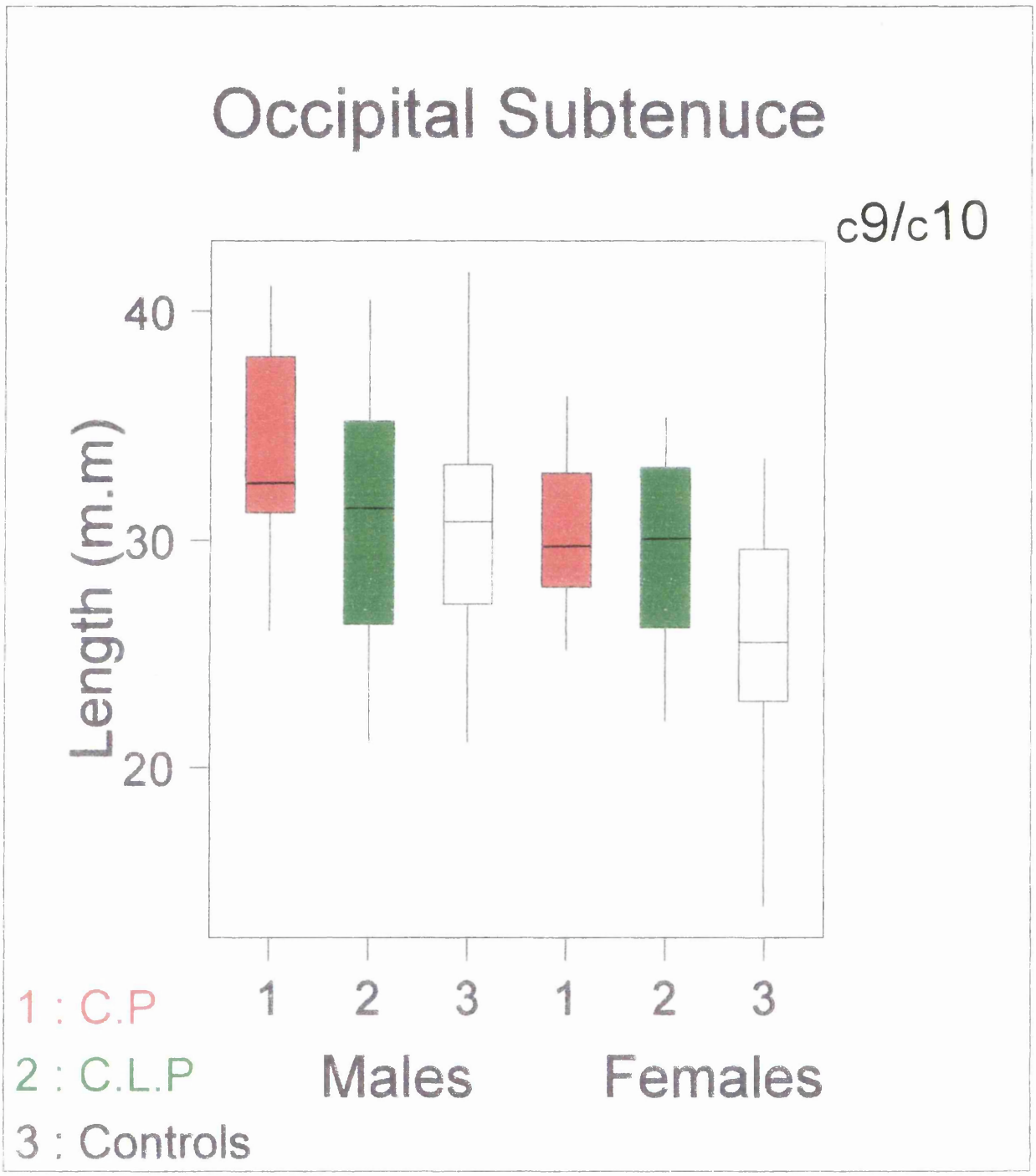

Cranial Height
(V - Ba)
c4
160
150
140
130
Length (m.m)
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls


Cranial Width
(Gla - Cpo)
c5
205
195
185
175
165
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females


Area of Cranium
c6
Area (c.m squared)
280
270
260
250
240
230
220
210
200
190
180
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls


Frontal Subtenuce
c7
Length (m.m)
30
25
20
1
2
3
1
2
3
Males
Females
1 : C.P
2 : C.L.P
3 : Controls


Parietal Subtenuce
c8
40
30
20
Length (m.m)
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females


Occipital Subtenuce
c9/c10
Length (m.m)
40
30
20
1 : C.P
2 : C.L.P
3 : Controls
1
2
3
1
2
3
Males
Females

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