## ADDENDUM

**A1. Description of the anatomical structures at a tissue and cellular level (pg 38).**

The description of the anatomy of the fetal membranes has been corrected.

Section 1.7.1.1 The paragraph should read:

"The placenta separates fetal and maternal blood. The trophoblast and the mesenchymal cells form the main cellular component of the chorionic villi. The trophoblast, which is discernible four days after fertilization, then differentiates into the outer and differentiated syncytiotrophoblast and the inner proliferative cytotrophoblast. As pregnancy progresses, fusion of cytotrophoblastic cells into the syncytiotrophoblast leads to a largely single-layered epithelium of syncytial character with only occasionally underlying proliferative cytotrophoblastic cells.

Section 1.7.1.2 should read:

"The amnion and the chorion are the fetal membranes, with the amniotic fluid the bridge between maternal decidua and the fetus. These membranes cover the placenta and separate the fetus from the mother. The membranes also maintain the fetus in a sterile and intact environment filled with amniotic fluid. The normal amnion is 0.02 to 0.5mm in thickness. The amnion, as well as an epithelial layer also has, according to Bourne (1962), a series of connective layers comprising basement membrane, a compact layer, a fibroblastic layer and a spongy layer. The amnion is in immediate contact with the amniotic fluid on one side and the underlying chorion on the other side. The chorion is a membrane composed of an outer syncytial layer without cellular divisions and an inner cellular (Langhans') layer. Macrophages are normally present in the layers of both amnion and chorion. The amnion and chorion, though slightly adherent, are never intimately connected and the amnion can be stripped from the top of the chorionic plate easily at term."

**A2. Use of serum pools**

The examiner makes an important point about the use of pooled rather than individual sera. He correctly points out that the rationale and limitations of this approach have not been adequately discussed. Accordingly, the following correction should have been inserted pp109, 2nd sentence of Discussion:

" To do this, pools of serum from healthy women with a singleton pregnancy were generated for fractionation. This was done because the limitations of the sensitivity of the fractionation methodology meant that the amounts of serum needed for just one inhibin fractionation experiment was high (at least 30mL). In order to meet this requirement, serum from individual patients had to be pooled as it was not possible to bleed sufficient women of at least 80mL of blood in order to obtain 30mL of serum. A limitation of this approach is therefore an inability to explore inter-subject variation in the inhibin forms present. However, this is not a critical limitation for two reasons. Firstly, it is unlikely that variation between subjects will be high because placental samples, which were fractionated individually, showed little variation in molecular weight sizes obtained (see Chapter Five). Secondly, the rationale behind these studies was to explore the potential for clinical application and therefore the observations have to be applicable to populations rather than individuals. Nonetheless, whether there is significant inter-subject variation in the inhibin forms present in maternal serum in normal pregnancy remains unknown and worthy of future examination."

**A3. Qualitative, semi-quantitative and quantitative (pg 91, 93)**

The examiner makes important points about the quantitative and qualitative nature of the data. Unfortunately, because the purification and fractionation procedures necessarily involve multiple steps, each with loss of

protein, the final data must be interpreted with this in mind. Nonetheless, the experiments described in Chapter Three carefully assessed the protein loss rates (expressed as recovery rates) for each step and for different protocols. In addition, the quality control runs undertaken ensured that these estimates of recovery were reproducible and of sufficient robustness to afford quantitative interpretation. Whether the data are expressed as absolute values, which are derived from arbitrary calibrators, or as recovered percentages and proportions of total recovered inhibin, they remain quantitative and the comparisons made between normal and abnormal profiles are valid. For instance, in figure 7.3, the mol wt profiles of inhibin A in normal and DS amniotic fluid shows that there is three-fold higher total inhibin A levels in normal pregnancies compared to aneuploidy, consistent with the findings of previous researchers (Wallace et al., 1997; Lambert-Messerlian et al., 1997). Quantitative assessments like these are made with all comparisons of normal with abnormal cases. The difficulties associated with using data complicated by varying recoveries are already discussed in Chapter 4, pp111-112.

**A4. Reliance upon the ELISAs**

The last discussion paragraph should have been moved to the end of chapter three, pp 91. In addition, a new sentence is added: "The assays utilized are those in commercial use and are the most sensitive and specific available at present. These assays have led to advances in understanding the physiology of inhibins, particularly in relation to the importance of the dimeric biologically active forms. However, it has been suggested that the current assays do not detect low mol wt forms of inhibin with sufficient sensitivity (David Robertson, personal communication), highlighting the need for the development of more specific assays. Certainly, as such assays come on-line further study of pregnancy sera and tissue would be worthwhile."

**B1. Append reprints of all published and in press papers arising from thesis**

pg 5 The list of publications is updated and reprints of all published and in press first-author papers are attached to the back of the thesis.

**P Thirunavukarasu**, T Stephenson, J Forray, PG Stanton, N Groome, E Wallace and DM Robertson. Changes in molecular weight forms of inhibin A and pro-αC in maternal serum during human pregnancy. *J Clin Endocrinol Metab* **86,** 5794-5804 (2001).

**P Thirunavukarasu** and EM Wallace. Measurement of inhibin A: a modification to an enzyme-linked immunosorbent assay. *Prenat Diagn* **21,** 638-641 (2001).

**P Thirunavukarasu**, DM Robertson, G Lambert-Messerlian, J Canick and EM Wallace. Inhibin A and pro-αC molecular weight forms in normal and aneuploid pregnancies. (In press, *Prenat Diagn*) (July, 2002).

**B2. Use of own references from work on thesis to justify other work in thesis**

pg 76 ln 28; pg 189 lns 15 &20 It is duly noted that references to my own papers should have been removed from the text and the reference list.

**B3. Other inhibin and activin forms**

pg 26 ln 11 The paragraph below gives more information on the formation of novel activins and inhibins with the other β subunits. "Five beta subunits have been isolated and characterized to date ($\beta_A$-$\beta_E$). The activin $\beta_C$ subunit was isolated from a human (Hotten et al., 1995) and mouse (Lau et al., 1996; Fang et al., 1996) cDNA library, the $\beta_D$ subunit from a Xenopus cDNA library (Oda et al., 1995) and the $\beta_E$ subunit from a mouse cDNA library (Fang et al., 1996). Pleiotropic functional activity has been ascribed to activin A, activin B and activin

AB, while the function of the $\beta_D$ subunit has been linked to mesoderm induction in Xenopus laevis embryos (Oda et al., 1995). The functions of $\beta_C$ and $\beta_E$ subunits however are not yet known. It also remains uncertain if the $\beta_C$-$\beta_E$ subunits assemble into homodimers, heterodimers or both. The current opinion is that the $\beta_C$ subunit is able to form dimers with the $\beta_A$ and $\beta_B$ subunits, but not with the α-subunit (Mellor et al., 2000)."

**B4. Value of ultrasound screening**

pg 50 1.8.2.3 The value of ultrasound is only dismissed as an inclusion to second trimester screening. I agree with the examiner that the measurement of nuchal translucency (NT) is a valuable screening marker but this is only of utility in the first trimester at 11-14 weeks of pregnancy rather than in the second trimester which was the focus of this particular paragraph.

**B5. Define Pregnancy Induced Hypertension**

pg 43 ln 10 The paragraph below clarifies the precise definitions: "Pregnancy-induced hypertension and pre-eclampsia are defined by the criteria set out by the World Health Organization and the International Society for the Study of Hypertension in Pregnancy (ISSHP) and recently up-dated by the Australian Society for the Study of Hypertension in Pregnancy (ASSHP) (Brown et al, 2000). Pregnancy-induced hypertension is defined as a) having a diastolic blood pressure of >90mmHg and b) non-proteinuric. Preeclampsia differs from PIH in the presence of significant proteinuria (>300mg/24hours). Preeclampsia is also typically of earlier onset than PIH and has multisystem involvement (eg liver, kidneys, heart, haematological, brain) not observed classically with PIH. In general, PIH is a more mild maternal condition than pre-eclampsia. Accordingly, at least part of the discrepancies between studies looking at the effects of PE on inhibin levels may reside in the inclusion of women with PIH. Therefore in Chapter Seven, only women exhibiting all of the criteria defining preeclampsia were included in the study on inhibin forms in preeclamptic women."

**B6. 'More defined' conditions for pooled samples should be better defined**

pg 97 ln 14 The stated 'more defined' conditions were with respect to collection, rather than gestation. The defining variation was in the treatment of the blood samples. Whereas blood samples that were 'normally collected' were stored at 4 C and centrifuged within 48h of collection, blood samples collected under 'more defined' conditions were collected on ice, stored at 4 C and centrifuged within 2h of collection. This is already mentioned in chapter four, pg 97, ln 14-19.

**B7. Interpretation of Figure 5.1**

Because of the differing scales of the y-axis between the graphs on page 118, it appears that the 75k inhibin A changes over the trimesters but it does not.

**B8. Ethnic variation**

There are no differences in the birth prevalence of Down syndrome across races. A paragraph inserted at the end of pg 164, ln 10 clarifies this: "There is no evidence to date that suggests different rates of Down syndrome in different ethnic groups."

**B9. Secretion of inhibin B by fetus into amniotic fluid**

pg 183 this was meant to read "However, there are no differences in amniotic fluid inhibin B levels between male and female babies, suggesting that secretion is through the chorion, and not through the fetus."

**B10. Staining**

pg 158 ln 9 "staining" was meant to read "immunocytochemical staining". Consideration of the limitations of this is now given: "In support of altered $\beta_A$ subunit production in DS, a recent study by Dalgliesh et al (2001) revealed stronger immunocytochemical staining of the $\beta_A$ subunit than the α-subunit in the placental trophoblasts and stroma of DS tissues. Importantly, while immunohistochemical staining is a semi-quantitative technique at best, it is reassuring that those data are consistent with the more quantitative tissue content and serum data reported in this Chapter and with the findings of other researchers (Wallace et al., 1996; Aitken et al., 1996; Lambert-Messerlian et al., 1996)."

**B11. Consistency in units and SI units**

Litres should have been abbreviated to L, not l, and milliliters to mL, not ml.

**Minor changes / typographical errors**

| | |
|---|---|
| Pg 9 ln 10 | the spelling of 'Celcius' is incorrect and should read 'Celsius' |
| Pg 10 ln 22 | μm should read μM |
| Pg 11 ln 9 | nm should read nM |
| Pg 11 | pro-αC should have been inserted into the abbreviations list "a processed form of the free alpha inhibin subunit" |
| Pg 28 Fig 1.2 | The line and asterisk is explained in the legend. "Inhibin α and α/β-subunit derived products in bovine follicular fluid. The asterisk atop a vertical line represents the position of N-linked glycosylation sites." The second glycosylation of human inhibin as discussed later is not shown in Fig 1.2 as the figure represents inhibin products identified in bovine follicular fluid only. |
| Pg 33 ln 26 | 'suite' should read 'suit' |
| Pg 38 ln 16 | 'peptide' should read 'peptides' |
| Pg 39 ln 20 | 'principle' should read 'principal' |
| Pg 41 ln 10 | the title 'Inhibins in fetal circulation' should read 'Inhibins in the fetus and fetal membranes' |
| Pg 43 ln 10 | 'this glycoprotein' should read 'it' |
| Pg 49 ln 27 | free βhCG should have been in the abbreviations list as "the free beta subunit of human chorionic gonadotrophin" |
| Pg 52 | a full stop should have been added to the end of the second paragraph |
| Pg 52 ln 27 | "centers" should read "centres" |
| Pg 57 | Title 2.3 should have been changed to "Procedures" |
| Pg 72 ln 2 | 'entirely mitigated' should read 'attenuated' |
| Pg 74 ln 12 | 'mitigated' should have been replaced by 'inhibited' |
| Pg 123 ln 5 | the spelling of syncytiotrophoblast has been corrected |
| Pg 182 ln 17 | IGF5 has been corrected to IGFBP5 |
| Pg 183 ln 14 | free αhCG should have been in the abbreviations list as "the free alpha subunit of human chorionic gonadotrophin" |

# STUDIES ON INHIBIN FORMS IN NORMAL AND ABNORMAL HUMAN PREGNANCY

*Prema P Thirunavukarasu*

BSc (Hons), The University of Melbourne

Department of Obstetrics and Gynaecology,
Monash University, Monash Medical Centre,
Clayton, Victoria, AUSTRALIA

A thesis submitted in accordance with the requirements for the Degree of Doctor of Philosophy

2001

## SUMMARY

Down syndrome (DS) is the single most common cause of intellectual handicap. Five years ago, the glycoprotein inhibin A was found to be increased in the maternal serum of DS pregnancies, suggesting utility as a prenatal marker. However, inhibin exists as multiple molecular weight (mol wt) forms in human biological fluids and no studies to date have examined the potential utility of specific inhibin mol wt forms as markers of pregnancy abnormalities. The focus of this work was to examine the molecular variants of inhibins in different pregnancy compartments of normal pregnancy, using an improved and validated immunoaffinity fractionation procedure, and to compare the proportions of form(s) with that in abnormal pregnancy, with the aim of improving inhibin as a prenatal marker of fetal and maternal pathologies.

Studies on normal pregnancy serum revealed that in addition to the 36k dimer, inhibin A mol wt forms of 66k, 32k and an abundance of forms <30k and pro-αC forms of 27k, 32k and forms less than <25k were also present in maternal serum. No inhibin B mol wt forms were identified in pregnancy serum. An increase in the proportion of inhibin A and pro-αC small mol wt forms (<30k and <25k, respectively) in late pregnancy was speculated to be due to cleavage by a late pregnancy blood protease.

Studies on normal placental tissues revealed that the major mol wt peaks of inhibin A and pro-αC in all three trimesters of pregnancy was 97-55k and 36-29k, representing partially processed or unprocessed dimers and processed or unprocessed free α-subunits, while studies on amniotic fluid revealed peaks of 36k and 66k inhibin A, 36k inhibin B and 29k and 32k pro-αC forms. The proportions of small mol wt forms of inhibins A and B (<30k) and pro-αC (<25k) in amniotic fluid were greater than that in matched maternal serum samples, suggesting differential sources of these small mol wt forms.

Studies on DS maternal serum and amniotic fluid and preeclamptic maternal serum showed no differences in either the inhibin species or the proportions of inhibin mol wt forms compared to normal. However, placental inhibin A forms and, to a lesser extent, placental pro-αC forms, were abnormally produced or cleaved in association with both Down syndrome and preeclampsia, suggesting that the placenta in abnormal

pregnancies undergoes altered processing of inhibin forms. These differences were not reflected in maternal serum suggesting that specific mol wt variants of inhibin A and pro-αC are unlikely to be useful markers of abnormal pregnancy.

In summary, the studies related in this thesis have afforded a comprehensive report on the inhibin forms present during normal and abnormal human pregnancy. While evidence is presented for altered placental production of inhibins in association with pregnancy pathologies, total inhibin A, rather than specific variants of inhibin A, was confirmed as the most discriminating marker of these pathologies.

## DECLARATION

The work embodied in this thesis was performed primarily at the Prince Henry's Institute of Medical Research and the Monash University Department of Obstetrics and Gynaecology in the period 1998-2001. This thesis contains no material accepted for the award of any other degree or diploma in any University nor materials previously published or written by any other person, except where due reference has been made in the text. All the work contained in this thesis was performed personally by the author with the exception of help indicated in the text of the thesis or under acknowledgements.

**Prema P. Thirunavukarasu**

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I thank Julie Forray for teaching me the gel chromatography procedure and assisting with initial validation studies, Tanneale Stephenson for performing the αIFMA assay and hFF fractionation and Dr Craig Harrison for allowing me the use of his mini-gel system and for his assistance with all things technical. I owe thanks to Lynda Foulds, who was a valuable source of advice on the procedure for processing placental tissue.

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This thesis is dedicated to my wonderfully supportive and loving parents, Arasu and Siva Nagalingam, and fiancé, Julian Finn. I owe them a million hugs for their years of financial support, encouragement and for their unwavering belief in me.

# PUBLICATIONS AND PRESENTATIONS

## Publications Arising from Thesis Studies

**P Thirunavukarasu** and EM Wallace. Screening for Down Syndrome: What is the best approach? *Perinatology* **1**, 79-84 (1998).

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**P Thirunavukarasu** and EM Wallace. Measurement of inhibin A: a modification to an enzyme-linked immunosorbent assay. *Prenat Diagn* (2001, in press).

**P Thirunavukarasu**, DM Robertson, G Lambert-Messerlian, J Canick and EM Wallace. Inhibin A and pro-αC molecular weight forms in normal and aneuploid pregnancies. (Submitted).

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**P Thirunavukarasu**, S Mulvey and EM Wallace. Ethnic variation in second trimester biochemical screening markers for Down syndrome. (Prepared for submission to *Prenat Diagn*).

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## ABBREVIATIONS

| | |
|---|---|
| **aa** | amino acid |
| **AcN** | acetonitrile |
| **AF** | amniotic fluid |
| **AFP** | alpha-fetoprotein |
| **ANOVA** | analysis of variance |
| **APS** | ammonium persulphate |
| **ASSHP** | Australian Society for the Study of Hypertension in Pregnancy |
| **AT** | 3-amino-1,2,4-triazole |
| **bSA** | bovine serum albumin |
| **°C** | degrees Celcius |
| **CI** | confidence interval |
| **$CO_2$** | carbon dioxide |
| **cpm** | counts per minute |
| **CRH** | corticotrophin-releasing hormone |
| **dbSA** | dialysed bovine serum albumin |
| **D&E** | dilatation and evacuation |
| **DNA** | deoxyribonucleic acid |
| **DR** | detection rate |
| **DS** | Down syndrome |
| **EDTA** | ethylenediaminetetraacetic acid |
| **EE** | electroelution |
| **ELISAs** | enzyme-linked immunosorbent assays |
| **FaST** | First and Second Trimester |
| **fβhCG** | free beta-subunit of human chorionic gonadotrophin |
| **FCS** | fetal calf serum |
| **FPR** | false positive rate |
| **FSH** | follicle stimulating hormone |
| **g** | gram |
| **GA** | gestational age |
| **g/litre** | grams per litre |
| **GnHCl** | guanidinium hydrochloride |

| | |
|---|---|
| **GnRH** | gonadotrophin-releasing hormone |
| **h** | hour |
| **hCG** | human chorionic gonadotrophin |
| **HCl** | hydrochloric acid |
| **HEPES** | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| **hFF** | human follicular fluid |
| **$H_2O_2$** | hydrogen peroxide |
| **HPLC** | high performance liquid chromatography |
| **IA** | immunoaffinity |
| **IFMA** | immunofluorometric assay |
| **IGF** | insulin growth factor |
| **IGFBP** | insulin growth factor binding protein |
| **IL** | interleukin |
| **ir-inhibin** | immunoreactive inhibin |
| **IRMA** | immunoradiometric assay |
| **ISSHP** | International Society for the Study of Hypertension in Pregnancy |
| **IU** | international units |
| **IVF** | in-vitro fertilization |
| **k** | kilodaltons |
| **kg** | kilograms |
| **µl** | microlitre |
| **LP** | luteal phase |
| **M** | molar |
| **µm** | micromolar |
| **mg** | milligram |
| **mins** | minutes |
| **mLs** | millilitres |
| **mM** | millimolar |
| **mmHg** | millimetres of mercury |
| **mol wt** | molecular weight |
| **MoM** | multiples of the median |
| **MQ** | double-deionised reversed osmosis water |

| | |
|---|---|
| **mRNA** | messenger ribonucleic acid |
| **MS** | maternal serum |
| **n** | number |
| **NaCl** | sodium chloride |
| **NADPH** | nicotinamide adenine dinucleotide phosphate |
| **$NaN_3$** | sodium azide |
| **NaOH** | sodium hydroxide |
| **nm** | nanometres |
| **nm/L** | nanomolars per litre |
| **NT** | nuchal translucency |
| **NTD** | neural tube defect |
| **OD** | optical density |
| **^** | power |
| **PAPP-A** | pregnancy associated plasma protein A |
| **PBS** | phosphate buffered saline |
| **PE** | preeclampsia/ preeclamptic |
| **pg/mL** | picograms per millilitre |
| **PIH** | pregnancy-induced hypertension |
| **PlGF** | placental growth factor |
| **pMSF** | phenylmethylsulphonylfluoride |
| **Prep-PAGE** | preparative polyacrylamide gel elution |
| **RCOG** | Royal College of Obstetricians & Gynaecologists |
| **RIA** | radioimmunoassay |
| **RP-HPLC** | reversed phase high performance liquid chromatography |
| **rpm** | revolutions per minute |
| **RT** | room temperature |
| **SD** | standard deviation |
| **SDS** | sodium dodecyl sulphate |
| **SDS-PAGE** | sodium dodecyl sulphate – polyacrylamide gel elution |
| **SE Asian** | South East Asian |
| **SEM** | standard error of the mean |

| | |
|---|---|
| **T13** | trisomy 13 |
| **T18** | trisomy 18 |
| **T21** | trisomy 21 (Down syndrome) |
| **TEMED** | tetramethyl-ethylenediamine |
| **TFA** | trifluoroacetic acid |
| **TGF-β** | transforming growth factor beta |
| **TNF** | tumour necrosis factor |
| **Tris-base** | tris(hydroxymethyl)-methylamine |
| **Tris-HCl** | tris(hydroxymethyl)-methylammonium chloride |
| **Tween-20** | polyoxyethylene(20)sorbitan monolaurate |
| **Tween-40** | polyoxyethylene(40)sorbitan monolaurate |
| **$uE_3$** | unconjugated estriol |
| **VCGS** | Victorian Clinical Genetics Service |
| **v/v** | volume per volume |
| **WHO** | World Health Organisation |
| **wks** | weeks |
| **wt** | weight |
| **w/v** | weight per volume |

## CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

# CHAPTER ONE

## Introduction: Studies on Inhibins In Normal and Abnormal Pregnancy

## 1.1 HISTORY

The existence of inhibin was first predicted in 1932 by Roy McCullagh while studying pituitary hypertrophy in rats with damaged seminiferous tubules. McCullagh (1932) reported the observation that administration of a water soluble extract of bovine testis prevented pituitary hypertrophy and he coined this as yet unidentified factor, 'inhibin'. Despite persistent efforts, he was unable to provide solid evidence for inhibin and there was little progress until the 1970s, when the development of radioimmunoassays permitted the measurement of FSH (Midgley, 1967). Support for the inhibin hypothesis was then offered by numerous studies showing that FSH could be selectively suppressed by various testicular and follicular fluids (Franchimont et al., 1972; Setchell and Jacks, 1974; Keogh et al., 1976). Indeed, much of the work was in testis, but the amount of activity in testis was rather low, hampering isolation attempts. A significant advance occurred in 1976 when it was reported that inhibin-like activity in follicular fluid was extremely high (de Jong and Sharpe, 1976). With the ready availability of large volumes of bovine follicular fluid in slaughterhouses, it was possible to isolate and partially characterize inhibin from this bovine source (Robertson et al., 1985, 1986; Fukuda et al., 1986). This was followed soon afterwards by inhibin characterization in porcine (Miyamoto et al., 1985; Rivier et al., 1985; Ling et al., 1985) and ovine (Leversha et al., 1987) follicular fluids.

It was during the numerous extraction and purification steps developed to isolate inhibin that the accidental discovery of a structurally related but functionally antagonistic protein, termed 'activin', occurred in the side fractions (Vale et al., 1986; Robertson et al., 1987a). Whereas inhibin inhibited FSH production and secretion, activin was found to stimulate FSH (Ying, 1988). Inhibin and activin were both postulated to regulate FSH secretion by endocrine feedback on the anterior pituitary gland. However, various studies showing mRNA for these factors expressed not only in the anterior pituitary itself but other tissues such as placenta, adrenal, kidney and spinal cord (Meunier et al., 1988) suggested that, in addition to regulating FSH secretion, these proteins were involved in more diverse biological functions. By the close of the 1980s, it was clear that inhibins and activins represented a significant new family of proteins, with diverse functions throughout mammalian reproduction. Investigation into the structure and function of these proteins commenced in earnest.

## 1.2 STRUCTURE OF INHIBIN

The purification of inhibin to homogeneity from several species (Ying, 1988; reviewed by de Kretser and Robertson, 1989) resolved the subunit structure of the molecule and this was confirmed by the crystal structure of inhibin, determined by X-ray crystallographic analysis (Burger and Igarashi, 1988; Daopin et al., 1992; Schlunegger and Grutter, 1992). Inhibin was revealed to be a dimeric molecule composed of two subunits, an α and a β, stabilized by a disulfide bond. Five β subunits have been reported ($\beta_A$, $\beta_B$, $\beta_C$ $\beta_D$, $\beta_E$) but only a single α subunit has been identified (Mason et al., 1985; Hotten et al., 1995; Oda et al., 1995). Thus, there is an extensive array of possible homo- and heterodimers, but of these, only $\alpha\beta_A$ (inhibin A), $\alpha\beta_B$ (inhibin B) and $\beta_A\beta_A$ (activin A), $\beta_A\beta_B$ (activin AB) and $\beta_B\beta_B$ (activin B) have been isolated as dimeric proteins in human tissue.

Sequence analysis of cDNA clones of inhibin from a number of species (Mason et al., 1985; Forage et al., 1986), including humans (Mason et al., 1986; Stewart et al., 1986), showed that the two subunits of inhibin were coded for by different genes, the gene products of which were large molecular weight precursor proteins. The α subunit is initially synthesized as a preproprotein and the β subunit as a proprotein (Figure 1.1). Inhibins and activins were subsequently classified as members of the transforming growth factor-β (TGF-β) superfamily (Kingsley, 1994) since they shared a common β subunit and the subunits of each mature dimer were encoded as the carboxyl-terminal parts of much larger precursor molecules. This superfamily includes TGF-βs (Massague, 1990), Mullerian inhibiting substances (Cate et al., 1986), bone morphogenic proteins (Wozney et al., 1988) and a protein coded for by the decapentaplegic gene complex in Drosophila (Padgett et al., 1987), important in morphogenesis. Although structurally similar, the TGF-β group is functionally diverse, being involved in a wide range of cellular processes including apoptosis, cell death, cell proliferation and differentiation (Massague, 1990).

Studies to date have determined the amino acid sequence of porcine (Mason et al., 1985), bovine (Forage et al., 1986) and human (Mason et al., 1986; Stewart et al., 1986) inhibin. These data have highlighted two main dibasic arginine proteolytic cleavage sites in the alpha subunit, dividing it into three regions: the 43-amino acid (aa) Pro region (composed of aa 19-61), the 171-aa αN region (aa 62-232) and the

134-aa αC region (aa 233-366) (Mason et al., 1985; Forage et al., 1986) (Figure 1.1). The inhibin $\beta_A$ subunit precursor consists of a 300-aa proβ region separated by five arginine residues from the C-terminal and the 116-aa $\beta_A$ region (Figure 1.1).

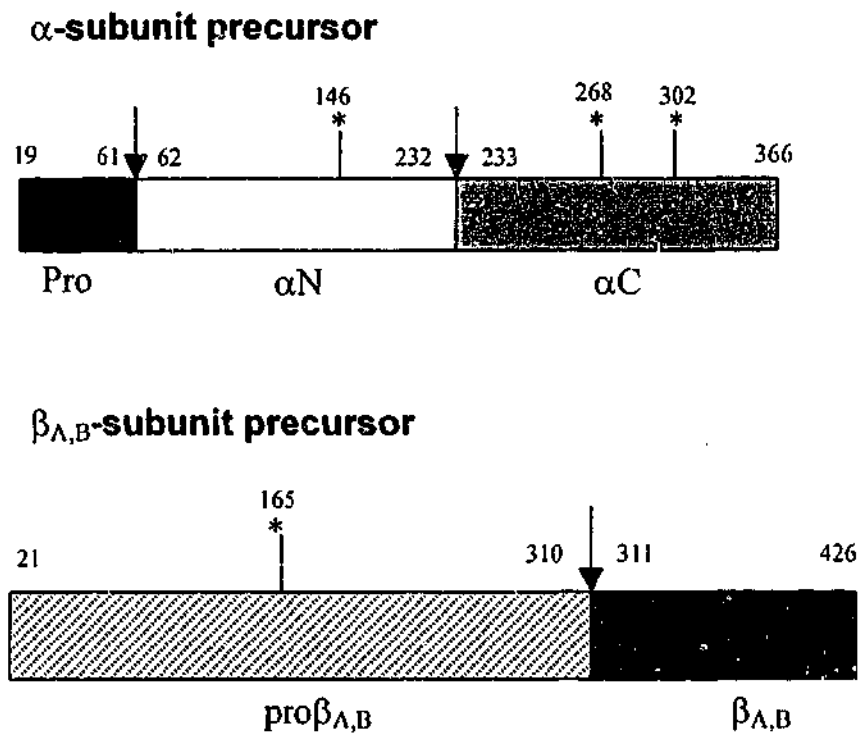



**Figure 1.1:** Diagrammatic representation of human α and $\beta_A$-subunit precursors. The positions of the processing sites in the precursors are indicated with arrows and the asterisks show the positions of the N-linked glycosylation sites. Numbering indicates amino acids numbers. (Modified from Mason et al., 1996a).

The α subunit in the two different forms of inhibin is common and there is 70% homology between $\beta_A$ and $\beta_B$ subunit amino acid sequences, with 30% homology between the sequences for the α and β subunits.

### 1.2.1 Inhibin molecular weights

The initial purification of inhibin from bovine follicular fluid by Robertson and colleagues (1986) revealed a 58k protein, consisting of the α and β subunits of 43k and 15k, respectively. The simple addition of an acidic pH precipitation step to the purification procedure resulted in the isolation of the smaller 32k species (Robertson et al., 1986), with this difference arising from cleavage of an $NH_2$-terminal segment of the α-subunit (Mason et al., 1985; Forage et al., 1986). Indeed, the α and β chains undergo subsequent processing, through several recognized steps, before forming the mature inhibin A or inhibin B form with a molecular weight of 31-32k. This cleavage

happens in the presence of serum, but not follicular fluid (McLachlan et al., 1986b), suggesting that processing of the α-subunit is extragonadal.

In previous studies of the isolation of inhibin from bovine and porcine follicular fluids, it was demonstrated that various molecular weight forms of inhibin existed, these forms relating to unprocessed and partially processed inhibin α-subunits, either free or dimerised to a β-subunit (Robertson et al., 1985; Miyamoto et al., 1986; Sugino et al., 1992; Good et al., 1995). The various molecular weight forms identified thus far are diagrammatically represented below.

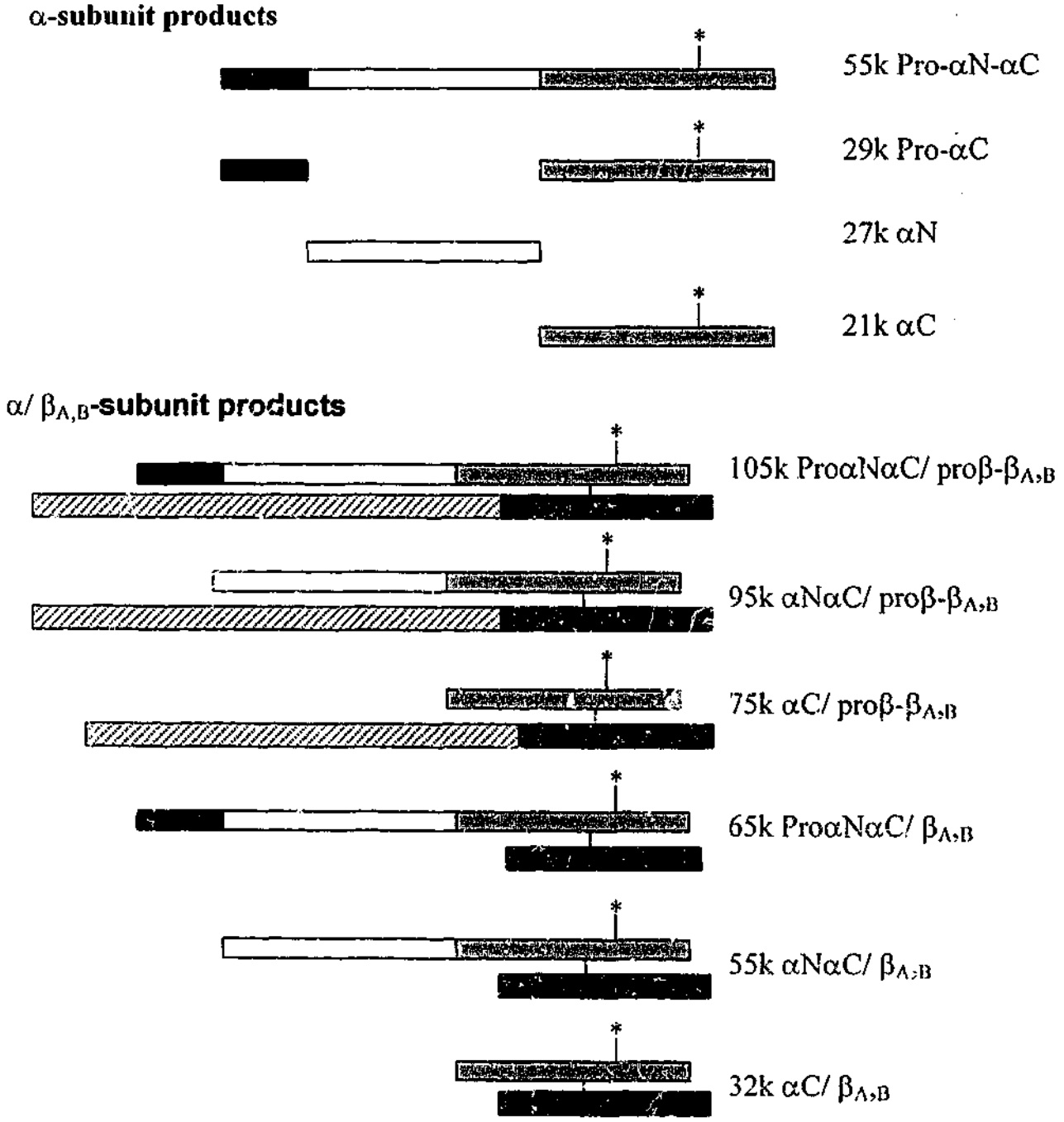



**Figure 1.2:** Inhibin α- and α/β- subunit derived products.

More recently, some of these forms have also been observed in human serum, both male and female (Robertson et al., 1995), and in human follicular fluid (Robertson et al., 1997a). A survey of the literature on inhibin shows that the molecular sizes assigned to each of the forms differ slightly between laboratories: for example, the 55k form is also described as 58k, and the 32k form is variously identified between 30k and 35k (McLachlan et al., 1986b; Miyamoto et al., 1986; Robertson et al., 1986; Muttukrishna et al., 1995). The studies in this thesis adopt the sizes initially suggested by Sugino et al (1992) for bovine inhibins, ie 32k, 55k, 65k, 75k, 95k and 105k, as that study presented the first systematic characterization of high molecular weight inhibin molecules. A notable difference between bovine and human inhibins is that, unlike the bovine αC-region of the α subunit, human αC may be glycosylated at one or two N-linked sites (Tierney et al., 1990), leading to the generation of both mono- and diglycosylated inhibin variants within each size class for human αC-containing molecules, with these forms differing by approximately 3k (Tierney et al., 1990).

Of all these forms, it is known that only the dimeric forms of inhibin are bioactive while the free α-subunits, which circulate in excess in biological fluids (Knight et al., 1989; Schneyer et al., 1990), do not possess FSH-suppressing activity (Knight et al., 1989, 1991; Robertson et al., 1989; Vale et al., 1990). More recently, Mason and colleagues (1996b) demonstrated that the full-length precursor $\alpha\beta_A$ dimer is inactive although, through alteration of the arginine-processing sites, the 65k and 55k forms of inhibin A are fully biologically active. However, precursor forms of inhibin are intrinsically less bioactive compared to 32k inhibin or inactive requiring cleavage of the precursor sequences for bioactivity to be evident (Robertson et al., 1997b). These findings suggest that understanding the basis of inhibin activity in serum will require an understanding of the distribution of inhibin forms as well as their individual biological activities. At this stage however, knowledge of the cell biology and physiology of the inhibin forms is far from clear.

## 1.3 BINDING PROTEINS

Two classes of soluble binding proteins exist for the activin/inhibin family of proteins: follistatins (Robertson et al., 1987a; Ueno et al., 1987; Michel et al., 1993) and $\alpha_2$-macroglobulins (Krummen et al., 1993). Like inhibin, follistatin was first identified based on its ability to suppress the biosynthesis and secretion of FSH by pituitary cells

(Ueno et al., 1987; De Paolo et al., 1991). However, although it is noted to have inhibin-like FSH suppressing activity (Robertson et al., 1987a; Ueno et al., 1987), follistatin is structurally unrelated to inhibin and activin. Further studies demonstrated that this molecule binds both activin and inhibin through their common β subunit (Nakamura et al., 1990; Kogawa et al., 1991; Shimonaka et al., 1991; de Winter et al., 1996), although at a much reduced affinity in inhibin compared with activin (Shimonaka et al., 1991; Schneyer et al., 1994), bioneutralizing the activity of activin in many tissues by forming a stable complex with it and preventing it from acting on distant target tissues (Woodruff, 1998). Binding to inhibin however does not appear to neutralize inhibin bioactivity.

$\alpha_2$-macroglobulin, another binding protein for inhibin and activin, is a broad-spectrum protease inhibitor known to bind a variety of growth factors, including TGFβ. α-macroglobulin is produced in the gonads and is present in blood (Mather, 1996). Although follistatin is probably the major inhibin/activin binding protein in the gonads, being present in high concentrations in human follicular fluid, $\alpha_2$-macroglobulin is present in greater abundance in serum, suggesting that it is a major binding protein of inhibin/activin in circulation. However, unlike follistatin, binding to $\alpha_2$-macroglobulin does not alter the bioactivity of inhibin or activin (Krummen et al., 1993). The action of $\alpha_2$-macroglobulin in serum may include altered clearance as $\alpha_2$-macroglobulin-bound inhibin and activin remain longer in the circulation than unbound forms (Krummen et al., 1993; Woodruff et al., 1993a, 1993b).

## 1.4 RECEPTORS

Inhibin-specific receptors have not yet been isolated or characterized, although recent progress has been made towards this goal (Woodruff, 1999). In contrast, two activin receptors have already been cloned and characterized (reviewed by Zimmerman and Mathews, 1996). Designated type I and type II, these activin receptors belong to a family of serine/threonine kinase receptors (Attisano et al., 1992; Ten Dijke et al., 1994) and, among various cell types, are expressed by granulosa cells, theca cells and oocytes (Eramaa et al., 1995; Mather et al., 1997; Sidis et al., 1998).

Binding of the activin ligand occurs via the activin type II receptor. This complex then interacts with the activin type I receptor, which itself has low intrinsic affinity for

activin. This union of type I and II receptors results in phosphorylation of the type I receptor which then phosphorylates nuclear activating factors, termed Smads, specific to the pathway (Kawabata and Miyazono, 1999), leading to nuclear activation.

The search for an inhibin-specific receptor has not been fruitful to date. However, Lewis and colleagues (2000) recently showed that inhibin binds with high affinity, via its α subunit, to the type III TGF-β receptor, betaglycan. This binding markedly enhances the binding of inhibin via its β subunit to the type II activin receptor. When transiently expressed in activin-responsive cells, betaglycan confers inhibin responsiveness to cells that otherwise respond poorly or not at all to inhibin. This suggests that at least some of the activin-opposing actions of inhibin probably involve interference with activin binding to its type II receptor, preventing activin-induced heteromerization of type I and type II receptors, a requirement for triggering the activin-dependent intracellular signaling cascade (Lebrun et al., 1997). It has been suggested by Knight and Glister (2001) that free inhibin α-subunit may compete with inhibin for binding to betaglycan, thus facilitating activin action by reducing the interaction of inhibin with the type II activin receptor. This hypothesis remains to be tested.

## 1.5 MEASUREMENT OF INHIBINS

### 1.5.1 Inhibin Bioassays

Prior to the development of radioimmunoassays, it was only possible to assess inhibin-like activity by observing changes in gonadal weight, reflecting FSH stimulation, under test conditions (Steelman and Pohley, 1953). Subsequently, the recognition of the existence of a specific regulator of FSH secretion from the anterior pituitary gland (Franchimont et al., 1972; Setchell and Jacks, 1974; Keogh et al., 1976) and the development of radioimmunoassays for FSH (Midgley 1967) afforded the development of other inhibin *in vivo* bioassays utilizing the *in vivo* suppression of FSH in castrated animals (de Jong and Sharpe, 1976; Marder et al., 1977). However, since large amounts of inhibin-containing materials must be used to obtain a response in *in vivo* methods, and the sensitivity of the pituitary gland to exogenous inhibin differs with the age of the animal (reviewed by de Jong, 1988), these *in vivo* methods are not practical.

The first *in vitro* assays for inhibin were bioassays using dispersed anterior pituitary cells (Vale et al., 1972). Inhibin was measured by assessing either the suppression of FSH secretion by pituitary cells into culture media (de Jong et al., 1979; Eddie et al., 1979; Lee et al., 1987) or the decrease in pituitary cell content of FSH (Scott et al., 1980). These procedures were largely insensitive to measuring inhibin in serum obtained under normal physiological conditions, although a subsequently developed *in vitro* bioassay, based on suppression of FSH release from ovine pituitary cell cultures has a much higher sensitivity and can be applied to serum (Tsonis et al., 1986).

The bioassay system provided a measure of inhibin bioactivity representing the total activities of inhibin, together with other hormones or growth factors, in the suppression of FSH release from cultured pituitary cells (Robertson et al., 1987a; Burger et al., 1993). Furthermore, the presence of the binding proteins, α-macroglobulin and follistatin, in human serum could alter the bioactivity of inhibin in bioassays. Therefore, the resulting 'inhibin' level was, in reality, a crude balance between inhibin and activin/follistatin bioactivity. *In vitro* systems might also be more sensitive to non-specific or toxic effects of added preparations, resulting in erroneous estimations of inhibin activity. Other bioassays utilizing the differential actions of inhibin and activin on human erythroid stem cell lines were also developed (Yu et al., 1987; Schwall and Lai, 1991) but they also reflected composite levels of the two proteins and other interfering substances.

### 1.5.2 Inhibin Radioimmunoassays (RIA)

The subsequent purification of inhibin in 1985 (Robertson et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985) allowed the generation of antibodies raised against either native proteins or synthetic peptides (Woodruff et al., 1994). A variety of assays were thereafter developed, the most useful of which was a radioimmunoassay developed by McLachlan and co-workers (1986b), known as the "Monash assay". This assay utilized a rabbit polyclonal antibody (antibody 1989) raised against 31k bovine inhibin, which recognized epitopes on the inhibin α-subunit (McLachlan et al., 1986b; Burger, 1992). It was rapidly applied to a variety of clinical situations, affording novel information about inhibin in both male and female reproduction (see section 1.6).

Such was the usefulness of this assay that much of the early literature on inhibin physiology is attributed to the Monash assay.

Subsequently, it became apparent that free α-subunits circulate in serum and that the Monash assay detected these (Robertson et al., 1989; Knight et al., 1989; Sugino et al., 1989; Schneyer et al., 1990). Indeed, the free α-subunit forms were the principal isoforms measured by the non-selective RIA (Schneyer et al., 1990). As mentioned previously (section 1.2), the free α-subunits are not thought to be biologically active and are of uncertain physiological relevance and thus interpretations of results obtained with α-subunit assays had to be necessarily cautious and limited (Burger, 1993). Furthermore, circulating binding proteins (follistatin and $\alpha_2$-macroglobulin), alter antibody-antigen binding in clinical samples such as plasma (Vaughan and Vale, 1993; Krummen et al., 1993) and apparent inhibin levels are altered by *in vitro* protease activity (Bramley et al., 1992). It was clear that the RIA was an inadequate tool to measure functional inhibin.

### 1.5.3 Immunoradiometric assay (IRMA)

In an attempt to overcome the major limitation of the RIA, Knight et al (1991) developed a sensitive and specific two-site immunoradiometric assay (IRMA) for dimeric inhibin. This assay showed negligible cross-reaction with either free α-subunit or the activin dimer (Knight et al., 1991). The specificity of the two-site IRMA for dimeric inhibin was attributable to the fact that two different antibodies were utilized, one recognizing an epitope on the fully-processed α subunit and the other recognizing an epitope on the mature β subunit. Other groups followed suite, using the same principle to develop two-site immunoassays for dimeric inhibin based on the used of enzyme-labelled antibodies rather than iodinated antibodies (like the Monash RIA) as tracers (Betteridge and Craven, 1991; Groome et al., 1991a; Baly et al., 1993). However, despite its high specificity for dimeric inhibin, the two-site IRMA was not sensitive enough to measure inhibin levels in peripheral or even ovarian vein samples.

### 1.5.4 Inhibin Enzyme-Linked Immunosorbent Assays (ELISAs)

Given the complexity of the inhibin family structure, with its precursors and free subunits, and the finding of the inhibin binding proteins, it became clear that new, more accurate assays were required. Several groups then tried preparing monoclonal

and polyclonal antibodies with the intention of producing two-site immunoassays to measure specifically the bioactive dimeric forms of inhibin (Baly et al., 1993; Poncelot and Franchimont, 1994). However, due to the high degree of structural conservation of inhibin between species, the immunogens were poor and the antibodies raised had limited affinity, making them unable to detect the picogram per milliliter concentrations in which inhibin circulates during the normal menstrual cycle (Groome et al., 1994).

Over the last few years, a group, led by Professor Nigel Groome, developed two-site immunoassays specific for inhibin A, inhibin B, activin A, activin B and α-subunit precursors (Groome, 1991a; Groome and O'Brien, 1993; Knight and Muttukrishna, 1994; Groome et al., 1995; Knight et al., 1996) which overcome the inherent limitation of the 'first generation' inhibin immunoassays developed in the mid to late 1980s, namely their tendency to cross-react extensively with 'free' α-subunits and their limited sensitivity. These new ELISAs clarified what were a number of apparent inconsistencies in data obtained with α-subunit based assays and resulted in an outpouring of important information on the control and ontogeny of the two mature forms of inhibin and the biological processing of these proteins. Indeed, our understanding of inhibin biology, initially based upon the Monash assay, has been considerably extended and modified by this new generation of assays.

### *1.5.4.1 Evolution of the inhibin ELISA*

The preparation of monoclonal antibodies raised against synthetic peptides, rather than native proteins, to the inhibin α-subunit (Groome et al., 1990) and subsequently the inhibin β-subunit (Groome and Lawrence, 1991b) allowed the development of a two-site immunoassay for inhibins A and B (Groome et al., 1994; 1996). Assay sensitivity sufficient for the application of the assay to human sera was achieved with modifications to the assay format (Groome and O'Brien, 1993), including a two-fold pre-assay sample treatment step that involved analyte denaturation and the addition of hydrogen peroxide prior to assay.

The peroxide step oxidizes the methionine residues in the β-subunit epitope (Knight and Muttukrishna, 1994) to improve reactivity with the respective antibody, thereby strikingly increasing assay sensitivity to such an extent that it allowed detection of as

little as 2ng/L of dimeric inhibin in human serum and plasma (Groome et al., 1994). The analyte denaturation step, initially introduced into the activin A assay to disrupt activin-follistatin complexes, (Knight et al., 1996), essential for the detection of activin, involved heating the sample or standard in a waterbath at 100°C for 3 minutes after mixing with sodium dodecyl sulphate (SDS), a detergent, to prevent the total denaturation of the protein. This step was incorporated into the inhibin assays as it was found to be effective in removing interfering substances (binding proteins, proteases, erythrocyte catalase) from the samples and heterophil antibodies in the inhibin B assay (Groome et al., 1996). Both steps were found to have a profound effect on the performance of the assay and are considered essential components of the overall procedure. This two-stage sample pretreatment step essentially overcame the inherent problem of inhibin-binding proteins by irreversibly disrupting activin/follistatin complexes (Knight et al., 1996). Details on the principle and protocols of the assays and a further modification is introduced in Chapter Two.

### *1.5.4.2 Sample source and stability*

Inhibin is relatively stable in both serum and plasma, although the former is more often used. As for some other analytes (Stenman et al., 1993), the chelating properties of citrate and ethylenediaminetetraacetic acid (EDTA) may have some influence on molecular weights of inhibin, particularly in late pregnancy (data suggesting this are described in Chapter Four). However, serum or plasma are appropriate for most quantitative inhibin A methods. As will be discussed further in Chapter Two, preservatives are not required, provided measurement is undertaken reasonably promptly and specimens are not kept for prolonged periods at room temperature.

An early study demonstrated that inhibin A is stable in unfrozen serum or plasma stored at room temperature for up to 2 days (Wallace et al., 1998b). A significant decline in inhibin A values was observed in whole blood stored at room temperature for only 2 days, attributed to interference by erythrocyte catalase, an enzyme released by red blood cells on lysing. The involvement of catalase in the inhibin A assay is investigated further in Chapter Two, which concludes with some important information for retaining stability of inhibin A levels in stored samples. In addition, stability of inhibin A levels during storage at –20°C and repeated freeze-thawing is also presented in Chapter Two. These observations indicate the need for care in

defining specimen requirements in maternal serum screening programmes, in which specimens may spend varying lengths of time in transit to the screening center, and in evaluating retrospective studies using stored specimens.

## 1.6 INHIBINS AND REPRODUCTION

The focus of this thesis is the measurement of inhibins in human pregnancy and so only a brief summary of inhibins in female reproduction is offered here with an obvious emphasis on inhibins, rather than activins.

While it was from the testis that inhibin-like activity was first demonstrated (McCullagh, 1932), and the testis is indeed the main source of inhibin in men (Ishida 1990), the understanding of the role of inhibin within the female gonads are much more well defined than they are within the male. The ovary is the main, if not sole, source of circulating inhibin in women of reproductive age (Illingworth et al., 1991), with secretion from the granulosa cells, or lutein cells in the corpus luteum, controlled by both gonadotrophins and various other factors, including insulin-like growth factor (IGF-1), TGFβ and activin (Tsonis et al., 1988; Hillier et al., 1991; Findlay, 1993; Hee et al., 1993).

The relative inhibin-contribution of each ovary is cycle-phase dependent (Illingworth et al., 1991), with both ovaries secreting similar amounts of inhibin during the follicular phase, whilst during the luteal phase, the ovary with the corpus luteum secretes the majority of inhibin. Circulating immunoreactive inhibin levels change throughout the menstrual cycle (McLachlan et al., 1987), with low inhibin in the early follicular phase (when FSH levels are falling) (Reddi et al., 1990), a transient peak with the midcycle LH/FSH surge and a steady rise to a maximum in the luteal phase (when FSH levels are low) (McLachlan et al., 1987). Studies using specific inhibin assays have related a similar pattern for inhibin A (Muttukrishna et al., 1994; Groome et al., 1994), with transient changes in inhibin B during the follicular phase when FSH levels are falling (Groome et al., 1996). The new assays also showed that the developing follicles secreted inhibin B preferentially, while inhibin A was the sole dimer secreted from the luteal cells (Groome et al., 1994; Muttukrishna et al., 1994). The different patterns of circulating inhibin A and B during the two phases of the ovarian cycle are strong evidence for their playing different physiological roles during

follicular recruitment, maturation and ovulation (Groome et al., 1996). What these roles are though remains unclear.

A large number of animal and human *in vitro* and *in vivo* studies have explored the roles of inhibins within the ovary (Ying et al., 1986; Hsueh, 1986; LaPolt et al., 1989; Woodruff et al., 1990; Hillier et al., 1991; Hillier et al., 1991; Xiao and Findlay, 1991, Xiao et al., 1992; Hillier and Miro, 1993; Findlay, 1993). Together these studies suggest that inhibin and activin, probably in consort with follistatin, ensure that there is both a mechanism of selecting the dominant follicle for ovulation, and of preventing premature luteinisation (Findlay, 1993; Baird and Smith, 1993). Inhibins appear to have important intra-gonadal paracrine/autocrine functions and a traditional endocrine role in the suppression of FSH secretion.

## 1.7 INHIBINS IN PREGNANCY

Human pregnancy is a physiological event that involves complex hormonal secretion and regulation from a variety of different gestational tissues including placenta, amnion, chorion and decidua. Placenta, amnion and chorion originate from the process of embryogenesis, while decidua originates from endometrial differentiation. These tissues interact to harmonise the complex processes involved in maintaining a uteroplacental environment which is favourable to both the fetus and the mother. From the time of implantation until the initiation of labour, placental tissues and fetal membranes release a range of steroid and protein hormones into fetal and maternal compartments which can act in an auto-, para- or endocrine fashion. These hormones include progesterone, oestrogens, chorionic gonadotrophin, placental lactogen, placental growth hormone, corticotrophin-releasing factor, oxytocin, prostaglandins, activin, inhibin, follistatin and a number of other pregnancy-associated proteins and molecules. The next few sections will give a brief introduction to the anatomy of the placenta and fetal membranes, followed by a discussion of inhibin in the maternal and fetal circulations.

### 1.7.1 Anatomical Structures

#### *1.7.1.1 Placenta*

The function of the placenta is three-fold: it serves to transmit nutrients to the fetus, to excrete waste products into the maternal blood and to modify maternal metabolism at various stages of pregnancy by means of its hormones. By the end of the first trimester of pregnancy, the human placenta achieves its mature architecture, with the functional unit the chorionic villus, consisting of a central core of loose connective tissue and abundant capillaries connecting it with the fetal circulation. Around this core is the syncytiotrophoblast, a continuous layer of cells which form the surface of the chorionic villi. These cells contain abundant cytoplasmic structures that are required for protein synthesis and secretion. The syncytiotrophoblast is in direct contact with the maternal bloodstream but is separated from fetal blood by a basement membrane, connective tissue and fetal vascular endothelium.

The developing fetus and its placenta form an interdependent partnership in regulating the endocrine-metabolic processes during the course of pregnancy. This functional relationship, commonly known as the fetal-placental unit, is a unique endocrine system that produces a large number of hormones, including peptide, neuropeptides, steroid hormones and peptide growth factors, many of which are identical with or at least mimic those produced by the hypothalamic-hypophyseal-target systems.

#### *1.7.1.2 Amnion and chorion*

Amnion and chorion are the fetal membranes. These membranes cover the placenta and separate the fetus from the mother. The membranes also maintain the fetus in a sterile and intact environment filled with amniotic fluid. The amnion is a double-layered translucent membrane. It consists of a single layer of cuboidal epithelial cells on a loose connective matrix. It is in immediate contact with the amniotic fluid on the one side and the underlying chorion on the other. The chorion is a membrane composed of an outer syncytial layer without cellular divisions and an inner cellular (Langhans') layer. Macrophages are present in the layers of both amnion and chorion. These tissues strip easily from the fetal surface of the placenta and can be separated by careful dissection.

### *1.7.1.3 Amniotic fluid*

At term, the fetus is bathed in approximately 1-2 litres of amniotic fluid. This fluid, formed mainly from fetal urine, protects the fetus from direct injury, aids in maintaining its temperature, allows free movement of the fetus, minimizes the likelihood of adherence of the fetus to the amniotic membrane and allows for hormonal, fluid and electrolyte exchange. Amniotic fluid functions as a repository for fetal secretions and excretions. Hormones and proteins like alpha-fetoprotein (AFP) and inhibin A are found in amniotic fluid. The formation of amniotic fluid is attributed to secretion of the amnion or fetal urine.

## 1.7.2 Inhibins in Maternal Circulation

Following the demonstration that the human corpus luteum secreted both dimeric inhibin and free α-subunit products (McLachlan et al., 1987b; Groome et al., 1994), inhibin levels were shown to rise in conception cycles in contrast to the declining levels in nonpregnant cycles (Lenton et al., 1991). Inhibin secretion is enhanced in pregnant women during gestation and decreases after delivery. The levels of circulating bioactive and immunoreactive inhibins increase progressively in maternal serum from 4-40 weeks and reach a peak at term (Qu and Thomas, 1998). After delivery, bio-and immuno-inhibins decrease markedly in maternal serum and become undetectable after 24 hours.

In humans, the principle bioactive form of inhibin in the circulation of pregnant women is inhibin A. In a study of the secretory profile of dimeric inhibins in different forms during early gestation, Illingworth and colleagues reported that the inhibin A concentration in plasma reached an initial peak on day 12 after ovulation (1996a). From day 21 after ovulation (five weeks' gestation), the inhibin concentration increased markedly and reached a peak on day 42 after ovulation (8 weeks' gestation), when the inhibin concentration was about five-fold higher than that during the normal menstrual cycle. Thereafter, the inhibin A concentration declined from 408 to 286 pg/ml 63 days after ovulation (11 weeks' gestation). In contrast, the inhibin B concentration was extremely low during early pregnancy. After ovulation the corpus luteum secretes inhibin A and it would appear to continue to do so into early pregnancy (Illingworth et al., 1996a). However, women without functional ovaries have similar immunoreactive inhibin levels in early pregnancy to normal women

(McLachlan et al., 1987b; Lenton et al., 1991; Santoro et al., 1992) suggesting that the ovary is not a major source of inhibin beyond six weeks of pregnancy. Indeed, a number of studies, reviewed by Qu and Thomas (1995), have suggested that the placenta is the main source of inhibins in pregnancy, as predicted by the first studies of placental extracts (Healy et al., 1988) and primary trophoblast cell cultures (Petraglia et al., 1987).

The control of inhibin secretion from the human placenta has not been fully elucidated but *in vitro*, hCG and GnRH stimulate trophoblastic inhibin secretion (Petraglia et al., 1987; Li et al., 1994) and reciprocally, inhibin suppresses hCG secretion. This latter effect is gestation-dependant since there is no inhibin-induced hCG suppression in cell cultures derived from first trimester trophoblast (Mersol-Barg et al., 1990). Inhibin may also regulate progesterone and prostaglandin production (Petraglia et al., 1993). Messenger RNAs for the three inhibin subunits, and the proteins themselves, have been localized to the syncytio – and cytotrophoblast (Petraglia et al., 1987; Petraglia et al., 1991; Minami et al., 1992; Rabinovici et al., 1992), the same cells secreting GnRH and hCG (Petraglia et al., 1992), with interesting changes in expression across gestations. In early pregnancy, mRNA for the α subunit is expressed in larger amounts than $\beta_A$ subunit mRNA, with very little $\beta_B$-subunit mRNA apparent (Petraglia et al., 1991). With increasing gestation, progressively more $\beta_B$ subunit mRNA is expressed suggesting that there is a gestation-dependent switch from a predominance of inhibin A and activin A secretion to the presence of many forms.

Circulating inhibin levels are significantly higher during pregnancy than in non-pregnancy (McLachlan et al., 1987b; Abe et al., 1990; Kettle et al., 1991; Tovanabutra et al., 1993). Plasma immunoreactive inhibin levels peak at 9-10 weeks, coinciding with the first trimester peak of hCG (Tovanabutra et al., 1993), and then fall to a plateau between 15 and 30 weeks, rising thereafter so that the highest levels are achieved by term (Abe et al., 1990; Tabei et al., 1991, Qu et al., 1991, Tovanabutra et al., 1993). After delivery, inhibin levels become undetectable within 4-5 days (Abe et al., 1990; Kettel et al., 1991). Interestingly, bioactive inhibin levels have been reported to rise steadily throughout pregnancy without the biphasic profile described for immunoreactive inhibin (Qu et al., 1991), consistent with a relative excess of inactive forms, either large mol wt dimers or, more likely free α-subunits, in early

pregnancy mirroring the relative excess of α-subunit message at this gestation. However using a specific assay, it was reported recently that maternal serum inhibin A levels display a biphasic profile (Muttukrishna et al., 1995; Fowler et al., 1998) indicating that the bioassay data were probably confounded by inadequate stripping of the very high levels of circulating sex steroids in the test samples (Qu et al., 1997) or by activins or other proteins that alter FSH secretion from the cells in culture (Petraglia et al., 1995). In support of the placental *in situ* and immunolocalization studies describing the ontogeny of subunit expression, inhibin B is undetectable in peripheral serum during pregnancy (Illingworth et al., 1996a; Wallace et al., 2000).

### 1.7.3 Inhibins in Fetal Circulation

While the placenta is the major source of inhibin secretion in pregnancy, the fetal membranes and the fetus have been suggested as other sources of inhibin, given the tissue distribution of the mRNAs for the inhibin subunits (Meunier et al., 1988; Petraglia et al., 1990; Tuuri et al., 1994). Riley and colleagues (1996) showed the presence of inhibin A, inhibin B and pro-αC in extra-embryonic coelomic fluid but only pro-αC in amniotic fluid in the first trimester of pregnancy (8-11 weeks' gestation). Indeed, only after 12 weeks' gestation, when the amniotic cavity has filled the intrauterine lumen, resulting in the fusing of the amnion and the chorion, are the dimeric inhibins then detected in amniotic fluid (Riley et al., 1996; Wallace et al., 1997a), suggesting that the fetus and fetal membranes are important sources of inhibins.

Importantly, Wallace and colleagues (1997a) have shown that in contrast to maternal serum, both inhibin A and inhibin B are detectable in amniotic fluid in the second trimester (Wallace et al., 1997a). Inhibin A in this compartment is significantly higher than in maternal serum (Wallace et al., 1997a) and studies have shown the lack of correlation of amniotic fluid inhibin A with maternal serum inhibin A (Wallace et al., 1997a). Taken with the findings of no inhibin B in maternal serum (Wallace et al., 1997a; Riley et al., 2000), this suggests the presence of at least two independent sources of inhibin.

The fetal membranes, in particular the chorion, is the likely source of inhibin A in amniotic fluid. Petraglia and colleagues (1993) demonstrated that the amnion selectively expresses $\beta_B$-subunit mRNA, which is more indicative of inhibin B production, but the chorion expressed mRNA for both the $\beta_A$- and $\alpha$-subunits, consistent with inhibin A secretion.

Sex differences are also apparent in inhibin levels. Neither inhibin A nor inhibin B are present in the cord serum from female babies, but in the males, levels of inhibin B are comparable to those in adult men (Illingworth et al., 1996b; Wallace et al., 1997a). The source of this inhibin B appears to be the fetal testis, which expresses mRNA for the inhibin subunits, whereas the ovary does not (Tuuri et al., 1994).

## 1.8 CLINICAL APPLICATIONS

In addition to examining the ontogeny of inhibins in normal pregnancy, a number of studies have recently assessed circulating levels of inhibin in a variety of pregnancy abnormalities. In pregnancies where it might be expected that placental mass would be reduced compared to normal, such as anembryonic pregnancies, ectopic pregnancies and threatened abortions, maternal serum inhibin levels, particularly pro-αC immunoreactivity, are lower than in normal pregnancy (Yohkaichiya et al., 1993, Illingworth et al., 1996a). It was therefore possible that inhibin would have a useful role in monitoring high risk first trimester pregnancies. This was assessed, and subsequently confirmed, by Lockwood and colleagues (1997) with the demonstration that inhibin A levels were low in pregnancies with embryonic failure. Immunoreactive inhibin levels are also higher in multiple pregnancies than in singletons (Yohkaichiya et al., 1993; Khalil et al., 1995) as well as being higher than normal in association with various pregnancy complications such as pre-eclampsia, fetal growth restriction or placental abruption (Khalil et al., 1995). These complications are all associated with disruption of the trophoblast basement membrane and there is a possibility that maternal serum inhibin may provide a basis for pregnancy complication screening.

Inhibin is also markedly elevated in molar pregnancy (Yohkaichiya et al., 1989) with an immunohistochemical study localizing the inhibin subunits in molar trophoblast (Minami et al., 1993) and suggesting that inhibin may have roles in molar

pathogenesis. However, it would appear that in a clinical setting, inhibin has nothing useful to add to hCG as a surveillance marker (Baddonel et al., 1994).

Potential clinical applications for inhibin A include polycystic ovarian syndrome (Roberts et al., 1994; Jaatinen et al., 1994; Cooke et al., 1995), ovarian cancer (Healy et al., 1993; Cooke et al., 1995; Burger et al., 1996), puberty studies as an indicator of maturity (Burger et al., 1988; Crofton et al., 1997; Byrd et al., 1998; Bergada et al., 2001) and IVF for monitoring the effects of gonadotropin stimulation (Lockwood et al., 1997; Treetampinich et al., 2000). Currently, the only application of inhibin A in clinical practice has been in prenatal screening for Down syndrome, where this glycoprotein has been shown to be significantly elevated (Wallace et al., 1994, 1995; Cuckle et al., 1995) to such an extent as to improve the detection rate in the second trimester (Aitken et al., 1996; Wenstrom et al., 1997). Inhibins are also poised to play an important role in the prediction of preeclampsia, a pregnancy-specific syndrome. The last few sections below focuses only on inhibins in preeclampsia and aneuploidy as inhibin A in these abnormalities are further investigated in this thesis. A more thorough introduction to prenatal screening for Down syndrome is also given below.

### 1.8.1 Preeclampsia

Hypertensive disorders of pregnancy, including preeclampsia and pregnancy-induced hypertension (PIH), are the most common of all pregnancy-related medical complications, affecting 5%-10% of pregnancies (Hsu et al., 1994) and are the most important single cause of maternal morbidity and mortality in the developed world (American College of Obstetricians and Gynaecologists, 1996). Because this condition is also commonly associated with significant fetal growth restriction (McCowan et al., 2001) and is a leading indication for preterm delivery, preeclampsia is also a leading cause of perinatal mortality and morbidity (Sibai et al., 1990; Roberts and Redman, 1993).

The signs and symptoms of preeclampsia are well described by the Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) (Brown et al., 2000). Diagnosis is determined by increased blood pressure accompanied by proteinuria (Perry and Beevers, 1994). Blood pressure increases by at least 30mmHg systolic or 15mmHg diastolic relative to blood pressure prior to 20 weeks' gestation. Proteinuria

is defined as the excretion of 0.1 g/litre of protein in a random specimen or 0.3 g/litre in a 24-hour specimen. Preeclampsia is also often characterised by altered renal function leading to salt and water retention and weight gain.

The management of preeclampsia has been compromised because of a poor understanding of the primary mechanism(s) underlying the disease and of the resultant injuries on the cardiovascular, renal, cerebral, haematological and placental systems. Indeed, the pathogenesis of this condition is not fully understood. However it is now widely accepted that vascular endothelial cell dysfunction is the final common pathway responsible for the maternal syndrome (Ness and Roberts, 1996; Dekker and Sibai, 1998). The underlying pathological changes that lead to the endothelial cell dysfunction remain incompletely understood, but poor placentation has been proposed as a major contributory factor (Roberts and Redman, 1993; Ness and Roberts, 1996). The theory is that placental ischemia results because of failure of the trophoblast to invade the spiral arteries in preeclampsia, which in turn leads to the release of one or more factors that are responsible for the damage of the maternal vascular endothelium (Roberts and Redman, 1993; Lyall and Greer, 1994). Since the normal process of trophoblastic invasion is complete by 20 weeks' gestation, the underlying placental pathology in preeclampsia must exist prior to this stage of pregnancy. This offers the exciting possibility that it may be possible to predict preeclampsia prior to clinical recognition.

Indeed, one of the key aims of modern antenatal care is to detect preeclampsia as early as possible, thereby allowing appropriate management. Various methods of predicting subsequent preeclampsia have been assessed, including Doppler flow ultrasound of the maternal and feto-placental circulations (North et al., 1994), vasoactive stress tests (reviewed by Dekker and Sibai, 2001) and the measurement of an array of proteins and peptides in the maternal circulation (Friedman et al., 1995; Perkins et al., 1995; Muller et al., 1996), however none of these approaches have not afforded adequate sensitivity and specificity. Therefore, the detection of preeclampsia continues to depend upon increasingly frequent antenatal visits in late pregnancy for blood pressure measurement and urinalysis. Unfortunately, while this approach is both costly and neither particularly sensitive nor specific, the modern obstetrician has little else to offer. Clearly, a sensitive, specific and non-invasive predictive marker for

preeclampsia would be a most important development and would afford the prospect of early targeted intervention and improved care. So far, non-specific treatments for preeclampsia have included adrenergic-blocking drugs (Magee et al., 1999), calcium antagonists (Magee et al., 1996), diuretic agents (Collins et al., 1985) and angiotensin-converting enzyme inhibitors (Hanssens et al., 1991). Also, a number of very general treatments have been suggested for the prevention of preeclampsia including calcium supplementation (Levine et al., 1997; Ritchie and King, 2000), aspirin (CLASP Collaborative Group, 1994; Christian, 1999) and vitamins C and E (Chappell et al., 1999). However, for such prophylaxis to be effective, it is imperative that any markers of preeclampsia are effective in predicting the disease relatively early in pregnancy. Recently, studies on inhibins and activins in preeclampsia have suggested that these proteins may be such markers.

Muttukrishna and colleagues (1997b) reported elevated concentrations of inhibin A and activin A in serum from women with severe preeclampsia compared with gestational age matched controls. Other investigators have also reported elevated inhibin A and activin A concentrations in preeclampsia (Petraglia et al., 1995; Fraser et al., 1998). These results are not entirely surprising given that the pregnancies complicated by preeclampsia are characterised by impaired placental development and function (Redman 1991) and it has been shown recently that inhibins/activins may regulate placental trophoblast invasion in early pregnancy (Canniggia et al., 1997). Since the normal process of trophoblastic invasion is completed by 20 weeks' gestation, it is of no surprise that levels of inhibin and activin would be greatly increased by this stage in women who subsequently developed preeclampsia.

Interestingly, the distinguishing feature of inhibin A and activin A as a potential marker of preeclampsia is that there may be little overlap between maternal serum levels in cases and controls, thereby affording excellent predictive sensitivity and specificity. In a study of women with established preeclampsia (Muttukrishna et al., 1997b), inhibin A levels were, on average, increased eight-fold and there was no overlap between cases and controls. In studies by Cuckle and colleagues (1998) and Muttukrishna and colleagues (2000), performed at 15-20 weeks' gestation, maternal serum inhibin A levels in the women who proceeded to develop preeclampsia were, on average, twice as high as controls but there was significant overlap between cases

and controls. These studies suggest that the degree of increase in inhibin A, and therefore the extent of overlap between cases and controls, alters with gestation and proximity to disease presentation. Better discrimination is certainly afforded with advancing gestation and proximity but this clearly offers less opportunity for prophylactic intervention.

In this respect, particular molecular weight forms of inhibin A, rather than 'total' inhibin A, may be more useful markers of preeclampsia. The potential for particular molecular weight forms of inhibin A to be useful predictive markers of preeclampsia has never been investigated. The studies in Chapter Six were conducted to investigate this further.

### 1.8.2 Down Syndrome

Nearly 100 years after the description of this condition by John Langdon Down in 1866, the cause of this syndrome was identified as an extra chromosome 21 (LeJeune et al., 1959). With an incidence at birth of approximately 1.3 per 1000, Down syndrome is the most common single cause of severe mental retardation in the developed world. Access to prenatal screening for Down syndrome has therefore become an important component of modern antenatal care (Royal College of Obstetricians and Gynaecologists, 1993; Roelofsen et al., 1993) and represents both a successful preventative public health measure and a facility highly valued by individual women. Clearly, the appropriateness and successfulness of any given screening programme for fetal abnormality will depend upon uptake by the population – itself a reflection of the local antenatal care culture and social and/or religious attitudes. However, these complex influences aside, there have been a number of significant developments in Down syndrome screening over recent years that have afforded a variety of different approaches to screening. The dilemmas about screening experienced by expectant mothers and their obstetricians have therefore been heightened by the availability of these new possibilities. This section of the review considers the recent developments, the various approaches to Down syndrome screening now possible and offers comment on what appears to be the best current practice. Particular emphasis is placed on the role of inhibin A in Down syndrome screening. Detailed accounts of the history of Down syndrome as a clinical condition can be found elsewhere (Smith and Berg, 1976; Zellwerger, 1977).

### *1.8.2.1 Maternal Age Screening*

Maternal age was the first prenatal screening test for Down syndrome. It has long been recognised that an individual's risk of having an affected pregnancy increased with her age (Fraser et al., 1876). The advent of successful karyotyping after second trimester amniocentesis (Jacobsen and Barter, 1967), and subsequently first trimester chorionic villus sampling (Simoni et al., 1983), thereby afforded an opportunity to utilize this knowledge to offer diagnosis to women over an arbitrary age. Maternal-age screening for Down syndrome was thus established. Women over the arbitrary cut-off age were deemed 'screen positive' and offered an amniocentesis while women younger than the cut-off age were 'screen negative' and offered no further testing.

Based largely on economic appraisals at the time, diagnosis was offered to 5% of the pregnant population, equivalent, depending on the population, to women aged 35-37 and over. Importantly, as the only risks available were those of having a liveborn Down syndrome baby, the maternal age at the time of delivery was used to identify those to be offered testing. Only subsequently has it become apparent that the majority of Down syndrome pregnancies are lost spontaneously (Halliday et al., 1995; Macintosh et al., 1996) and therefore that the risks vary considerably with gestation. However, the important risk remains the risk at delivery and this would appear to be the most appropriate risk to use when counseling women or couples, irrespective of the gestation at counseling.

Using such an approach to screening, 5% of pregnant women would be offered testing and 30% of Down syndrome pregnancies would be detected. While this approach of maternal age-directed diagnosis continues to be popular today, guiding both first and second trimester testing, these figures emphasise that while the risk of having an affected pregnancy increases with maternal age, the majority of Down syndrome babies are born to women under the age of 35. Maternal age screening, on its own, is therefore not an effective approach.

### *1.8.2.2 Serum Screening*

Down syndrome pregnancies are associated, on average, with abnormal maternal serum levels of a number of biochemical markers (Merkatz et al., 1984; Bogart et al.,

1987; Canick et al., 1988). This association was first observed for alpha-fetoprotein (AFP), which was being used as a serum marker for neural tube defects (Brock and Sutcliffe, 1972). AFP is a glycoprotein with a molecular weight of 69k. In the fetus, AFP is synthesized by the liver, the gastrointestinal trac' and the yolk sac (Gitlin and Perricelli, 1970; Bɛlanger et al., 1982). While the yolk sac is the major contributor of AFP early in gestation, it is replaced by the liver as pregnancy progresses. In the mother, circulating AFP levels rise progressively to reach a peak at 32 weeks gestation, then decreases towards term (Leek et al., 1975). The mechanisms that control the synthesis of AFP are unknown as are its functions.

In contrast to pregnancies complicated by a neural tube defect where maternal serum AFP in increased, second trimester maternal serum AFP in pregnancies with a Down syndrome fetus are, on average, decreased by approximately 30% (Merkatz et al., 1984). Subsequently, the evaluation of a number of feto-placental products led to the identification of other possible second trimester biochemical serum markers. Importantly, maternal age remains a useful and independent variable such that the biochemical markers are used to adjust the age-related prior risk. Today, most serum screening programmes are based upon maternal serum levels of AFP and human chorionic gonadotrophin (hCG) with or without unconjugated estriol ($uE_3$). hCG is a heterodimeric glycoprotein produced by the syncytiotrophoblast of the placenta (Kurman et al., 1984) with the α subunit synthesized in greater quantities than the β-subunit (Boothby et al., 1983). In biological fluids, hCG may exist as intact heterodimeric hCG (dimer of two non-covalently linked α and β subunit), as modified hCG or 'nicked' hCG, so called because of missing linkages in the β40-50 region, as its free subunits (α or β) or as degradation products (β-core fragment) (Birken et al., 1996). This provides some dilemma over which measurement is optimum for screening abnormalities: while some investigators dispute the superiority of serum hCGβ over intact hCG (Wald et al., 1993; Cuckle et al., 2000), others argue that urinary hCGβ-core fragment are much more increased in urine than hCGβ concentrations in blood (Cuckle et al., 1994a; Iles, 1996) and would therefore make better markers. This remains to be determined and would first require improvements in both methodology and assay standardization.

The three serum markers (AFP, hCG and $uE_3$), together with maternal age, formed the basis of the so-called 'triple test'. Extensive evaluation of this approach has demonstrated a detection rate of 60-70% for a 5% false positive rate (FPR) (Wald et al., 1992a; Royal College of Obstetricians and Gynaecologists, 1993) in many different populations. Furthermore, the use of the free β-subunit of hCG, in preference to intact hCG, and inhibin A instead of $uE_3$ increases the detection rate yet further for a fixed 5% FPR (Spencer et al., 1992, Aitken et al., 1996). Indeed, inhibin A has now been shown to be very useful in the detection of Down syndrome, although this was not always the case. Initial studies of inhibins in Down syndrome pregnancies used inhibin assays that measured both dimeric inhibin and free inhibin α-subunits. These results suggested that maternal serum levels of immunoreactive inhibin (ir-inhibin) were, on average, elevated in pregnancies with a Down syndrome (DS) fetus (Van Lith et al., 1992; Spencer et al., 1993). However, the degree of discrimination between DS and unaffected pregnancies afforded by ir-inhibin was not sufficient for it to be a clinically useful serum marker (Spencer et al., 1993; Cuckle et al., 1994b). The possibility that the degree of discrimination might depend on the specificity of the inhibin assay was first suggested by Wallace and colleagues in 1994 when, after measuring ir-inhibin in first trimester maternal serum from 11 DS pregnancies and 89 controls using two different immunoassays, they found a poor correlation between the two, with the results derived from one assay failing to discriminate between the DS and control pregnancies.

The subsequent development and application of a sensitive and specific immunoassay for inhibin A (Groome and O'Brien, 1993) confirmed these earlier suggestions by Wallace and colleagues (1994), with several investigators showing that inhibin A was significantly more elevated than ir-inhibin in the same DS samples (Cuckle et al., 1994b; Cuckle et al., 1995; Wallace et al., 1995). It is now well established that inhibin A is significantly elevated in DS samples in the second trimester of pregnancy (Cuckle et al., 1995; Wallace et al., 1996; Lambert-Messerlian et al., 1996a; Noble et al., 1997). Indeed, inhibin A increases the prenatal detection rate of DS by up to 20% when added to existing prenatal serum markers (Aitken et al., 1996; Cuckle et al., 1996; Wenstrom et al., 1997). Second trimester screening using maternal age with maternal serum AFP, fβhCG and inhibin A, with or without $uE_3$, therefore appears to be the optimum current combination.

### *1.8.2.3 Second trimester ultrasound screening*

Down syndrome is also associated with a number of structural abnormalities, many of which are apparent with ultrasound (Nicolaides et al., 1992) and the presence of one or more of these in a fetus is an indication for karyotyping. Such defects include duodenal atresia, cardiac anomalies, cerebral ventriculomegaly, nuchal oedema, pyelectasis, echogenic bowel, shortened femora and varied combinations. However, despite these multiple abnormalities, and the need to consider karyotyping in an individual pregnancy with an identifiable abnormality, a Down syndrome screening programme based upon second trimester ultrasound will detect only 40% of Down syndrome fetuses, approximately comparable with screening by maternal age alone and significantly worse than second trimester serum screening. Furthermore, ultrasound detection is not additive to biochemical screening (Owen et al., 1994). There is therefore no place for a prenatal Down syndrome screening programme based solely upon second trimester ultrasound. This of course does not detract from the utility of ultrasound to detect structural abnormalities, although even this has been brought into question (Ewigman et al., 1993).

### *1.8.2.4 First trimester screening*

There are obvious advantages to offering screening and diagnosis of Down syndrome in the first trimester of pregnancy. Indeed, a desire for early diagnosis is currently reflected by the many older women who currently opt for chorion villus sampling at 10 weeks or so in preference to an amniocentesis later. As in the second trimester, both biochemical and ultrasound screening is possible in the first trimester. While many biochemical markers have been assessed for use in the first trimester, the most promising are fβhCG and pregnancy-associated plasma protein A (PAPP-A). Levels of the former are elevated approximately two-fold in association with Down syndrome, as they are in the second trimester, while PAPP-A is profoundly depressed (Krantz et al., 1996). PAPP-A is not a discriminating marker in the second trimester. Initial retrospective studies suggested that first trimester serum screening based upon these markers, in combination with maternal age, could afford a detection rate of 60-65% for a 5% FPR (Aitken et al., 1993; Krantz et al., 1996), similar to second trimester screening. Very recently, a prospective study in a high risk (older) population has confirmed these performance predictions (Haddow et al., 1998),

although lower detection rates would be expected in a general population. In a small study of first and second trimester screening in the same population, Berry and her colleagues (1997) have shown that second trimester screening (AFP/intact hCG) afforded a higher detection rate than PAPP-A/fβhCG. While first trimester serum screening looks most promising therefore, the available evidence would not support replacing optimized second trimester screening (AFP/fβhCG/inhibin A) at present.

In contrast, first trimester ultrasound screening using a measurement of nuchal translucency (NT), thought to be due to excess nuchal fluid accumulation in Down syndrome fetuses, has been shown to be a powerful indicator of increased risk of aneuploidy (Snijders et al., 1996) and significantly better than second trimester ultrasound. In large prospective series, in both high and low risk populations, Nicolaides and his colleagues (1997) have used NT to screen for Down syndrome, consistently achieving a detection rate of approximately 80% for a 5% FPR (Noble et al., 1995). These data would suggest that NT may be the most effective single prenatal marker of Down syndrome yet described. Furthermore, NT and biochemical markers are independent from each other and the addition of first trimester serum markers may increase the DR to 85-90% for a fixed 5% FPR (Mulvey and Wallace, 2000a). Nuchal translucency, with or without biochemical markers, would therefore appear to represent a significant improvement on second trimester serum screening, irrespective of the second trimester marker combination used.

However, most groups reporting their experience of NT have been unable to achieve such exciting results (Hewitt et al., 1996; Scott et al., 1996; Kornmann et al., 1996; Haddow et al., 1998). Indeed, some of these studies have recorded detection rates significantly poorer than achievable with second trimester serum screening. While it is currently unclear why these differences exist, and it may simply be due to different methodologies in measuring NT, without local evaluation it would be premature for NT to find widespread acceptance as the preferred method of Down syndrome screening.

In addition, there are a number of issues related to first trimester screening and diagnosis per se that merit further consideration. Firstly, chorionic villus sampling or early amniocentesis are associated with a procedure-related loss rate higher than that

following second trimester amniocentesis (Alfirevic et al., 1997) and an abnormal placental karyotype will be detected in 1-2% of chorionic villus samples, requiring further diagnosis, and risk of pregnancy loss, to define whether this is placentally confined. It would be important to discuss these possibilities with a woman or couple prior to choosing the most appropriate diagnostic method for them.

Furthermore, approximately two thirds of Down syndrome pregnancies are spontaneously lost, with an estimated 50% miscarrying between 10 and 16 weeks gestation (Macintosh et al., 1996). Thus, many affected pregnancies detected by screening and diagnosis in the first trimester may have aborted spontaneously before second trimester screening. Indeed, there is evidence to suggest that both biochemical and NT screening will selectively identify pregnancies at risk of spontaneous loss (Hyett et al., 1996). This is important for two reasons. Firstly, some parents with a Down syndrome pregnancy will be forced to face, unnecessarily, the decision and anguish of whether or not to terminate their pregnancy. Secondly, there will be an unnecessary burden of health costs associated with counseling and then termination and follow-up of the women who would have miscarried. It would therefore be important to quantify the risk of miscarriage in the pregnancies detected by early screening. Such information would be critical in counseling and would allow individual women to choose the timing and method of screening and/or diagnosis most suitable for them. Nonetheless, current data (Mulvey and Wallace, 2000b) suggests that a majority of women still express a clear preference for first trimester screening tests for Down syndrome

Current second trimester serum screening also screens effectively for neural tube defects, using maternal serum AFP as a marker (Wald, 1995). Unfortunately, AFP is not a useful marker of neural tube defects in the first trimester and if second trimester serum screening was replaced with first trimester screening, whether biochemical and/or ultrasound, in those centers with a high incidence of neural tube defects some consideration would still have to be given for NTD screening, or diagnosis by ultrasound, in the second trimester. It would therefore be important for such centers to assess the local costs of separate Down syndrome and NTD screening programmes with those of the unified approach currently popular.

In conclusion, it should be clear therefore that while earlier screening is highly desirable, until the performance of the first trimester approaches, relative to second trimester serum screening, are clearer the available evidence would not support their widespread uptake into clinical practice. Until directly comparable data are available, second trimester biochemical screening for Down syndrome, using a combination of AFP, βhCG and inhibin A, remains the most evaluated and valued approach.

## 1.9 SUMMARY AND AIMS

All reports of inhibin A in screening for preeclampsia and Down syndrome to date have focused on 'total' inhibin A, that is, the combination of the 32k mature dimer and different sized variants of inhibin A, obtained as a result of differential post-translational processing, particularly of the α subunit. The potential for particular molecular weight forms of inhibin A to be useful markers of these abnormalities has been largely ignored, possibly because of the conflicting results from two studies using chromatographic analysis of normal human pregnancy sera (Muttukrishna et al., 1995; Khalil et al., 1995). One study suggested that only the fully processed 32k inhibin was present in maternal serum (Muttukrishna et al., 1995). However, another report the same year (Khalil et al., 1995) described very little 32k inhibin in maternal serum but instead a predominance of 55-60k forms. Both studies were performed using immunoblot analysis, a technique with limited resolution. Given that immunoaffinity fractionation by Robertson and colleagues (1996, 1997a,b) revealed multiple molecular weight forms of inhibins in human follicular fluid, IVF serum, postmenopausal serum and male plasma, with the forms differing in the different fluids, it was likely that a similar scenario existed for inhibins in the different compartments of human pregnancy. The studies presented in this thesis were primarily undertaken to establish which molecular weight species of inhibin A, inhibin B and pro-αC were present in normal human pregnancy using a modified and validated immunoaffinity chromatography procedure with better resolution than the procedures used by Khalil and colleagues (1995) or Muttukrishna and colleagues (1995). The underlying rationale of course was to improve the utility of inhibin A as a marker of abnormal pregnancies by identifying which molecular sizes of inhibin were particularly deranged in cases of Down syndrome and preeclampsia compared to controls. If differences do exist, then the implications for screening would be significant, and would encourage the development of new antibodies against specific

epitopes, allowing the detection of specific forms of inhibin A and therefore offering a further improvement in diagnosing preeclamptic and Down syndrome pregnancies.

The specific aims of this study were therefore to:

1. Validate and improve the current inhibin A assay (Chapter Two).
2. Identify inhibin A, inhibin B and pro-αC inhibin molecular weight forms in pregnancy serum, using a modified immunoaffinity fractionation procedure. (Chapters Three and Four).
3. Identify the inhibin molecular weight forms in placental tissues (Chapter Five).
4. Identify and compare inhibin molecular weight forms in preeclamptic and normal pregnancies (Chapter Six).
5. Identify and compare inhibin molecular weight forms in normal and aneuploid pregnancies (Chapter Seven).
6. Test the extent that race/ethnicity affects the performance of 2$^{nd}$ trimester biochemical markers of Down syndrome, and whether allowances should be made for this factor, thereby improving the performance of the screening programme (Chapter Eight).

The data presented in the following pages offer, for the first time, the comprehensive report on inhibin molecular weight forms in pregnancy to date.

# CHAPTER TWO

## Inhibin ELISAs: Measurement & Modifications

## 2.1 SUMMARY

This chapter describes in detail the principles and protocols of the different inhibin immunoassays used in the studies reported in this thesis. Validation studies conducted confirm that, for one inhibin A ELISA format, short term sample storage as whole blood leads to a significant decline in detectable inhibin A and that this is most likely due to erythrocyte catalase interference with a critical oxidation step in the assay. While this interference can be eliminated by heating the samples pre-assay, this process is labour intensive and not conducive to high throughput. The studies detailed in this chapter demonstrate that the addition of 3-amino-1,2,4-triazole (AT), a catalase 'suicide' inhibitor, also prevents the decline of inhibin A levels in samples stored as whole blood. It is suggested that the addition of AT to samples prior to assay is a simple modification to the inhibin A ELISA that affords optimum performance.

## 2.2 INTRODUCTION

Since the original description of the enzyme-linked immunosorbent assay (ELISA) for inhibin A (Groome and O'Brien, 1993), a number of minor modifications have been introduced. One of those modifications involves the pre-assay addition of sodium dodecyl sulphate (SDS) and heating of samples at 100°C for three minutes (Knight and Muttukrishna, 1994). This format was shown to optimise assay performance and, more importantly, afford improved discrimination between Down syndrome samples and controls (Wallace et al., 1998b). This step is therefore recommended in the current product insert of the commercially available inhibin A assay (Oxford Bio-innovation, Upper Heyford, UK). However, pre-assay sample heating is labour intensive and does not lend itself easily to automation. Understandably therefore, many laboratories continue to perform the assay without the heating modification. Clearly, a simpler alternative to this pre-assay heating step is needed.

The first part of this chapter (sections 2.3-2.4) focuses on a formal assessment of the performance of inhibin assays in clinical samples. These sections detail the principles and protocols of the inhibin assays. As the samples collected for the studies detailed in the next few chapters had variable times of storage at room temperature before processing, and were performed using archival amniotic fluid and sera, some of which had been freeze-thawed on more than one occasion, assessments of the effects of storage and freeze-thawing on inhibin levels were also conducted. The second part of this chapter (sections 2.5-2.7) explores storage effects further, providing a further assay modification.

## 2.3 PRINCIPALS & PROTOCOLS

Information about the reagents and materials used for the ELISAs, and their sources, are presented in the Appendix.

### 2.3.1 Assay Solutions

a. ***Pro-αC diluent***

Add 12.10g Tris and 8.8g NaCl to 1000mls 0.1M HCl. Adjust pH to 7.5 with NaOH. Remove 100mls and add 50mls of triton X100 (5% w/v), 100g bSA (10% w/v), 50mls normal mouse serum (pre-filtered 0.4µl filter) and 1g $NaN_3$.

***b. Assay diluent***

Add 12.10g Tris and 8.8g NaCl to 1000mls 0.1M HCl. Adjust pH to 7.5 with NaOH. Remove 50mls and add 50mls of triton X100 (5% w/v), 100g bSA (10% w/v), and 1g $NaN_3$.

*c.* ***Sodium dodecyl sulfate (SDS) solution (6% w/v):***

Add 6g of SDS to 100mls distilled $H_2O$.

*d.* ***$H_2O_2$ (15%v/v):***

Add 3mL stock hydrogen peroxide (30%) to 3mL distilled $H_2O$.

*e.* ***Wash buffer solution***

Add 52.62 g NaCl, 36.36g Tris, and 3mls of Tween 20 to 5.5L distilled $H_2O$. Using HCl, adjust to pH 7.5 and make up to 6L with distilled $H_2O$. Store at room temperature.

*f.* ***Fab R1 alkaline phosphatase conjugate (1:200 dilution):***

For 2 plates, add 60µl of R1 to 12mls of solution 2.

*g.* ***Fab R1 alkaline phosphatase conjugate (1:100 dilution for Pro-αC assay):***

For 2 plates, add 120µl to 12 mls of solution 1.

***h. Stop Solution (0.4M HCl):***

To 1L distilled water, add 34.4mL concentrated HCl.

### 2.3.2 Inhibin A ELISA

96-well microtitre plates (Nunc, Maxisorb), pre-coated with a biotinylated (E4) monoclonal antibody (Oxford Bio-innovation, UK) (Groome and Lawrence, 1991b) raised against the $\beta_A$-subunit peptide (amino acids, (aa) 82-114) of inhibin A, were used throughout. Samples, standards and quality controls were incubated in the wells so that the antigen bound to the 'capture' or 'immobilised' antibody via its $\beta_A$-subunit. Following manual washing of the plate with wash buffer, a 'second' or detection antibody (Fab R1) was added. This is a mouse monoclonal antibody specific for the α-subunit peptide (aa 1-32) of inhibin (coupled to alkaline phosphatase). Any

unreacted material was then removed by washing before the detection of the alkaline phosphatase using a sensitive amplified substrate reaction (ELISA Amplification System, Gibco-BRL, Melbourne, Australia). In brief, this involved addition of 50ul substrate, which contains a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), that acts to conjugate to the bound inhibin A. An amplifying solution containing lyophilised alcohol dehydrogenase and diaphorase was then added, acting to dephosphorylate NADPH to NADH, which in turn activates secondary enzymes to reduce a tetrazolium salt to form an intensely coloured 'formazan' dye. This dye is itself oxidized to form NAD+. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of NADH originally formed by the enzyme in the bound conjugate. Thus, the colour intensity reflects the concentration of inhibin present.

Before the ELISA, patient samples and standards are pre-treated with detergent (SDS) and heated to 100°C before being exposed to hydrogen peroxide. Heating samples with an SDS solution enhances the sensitivity of the assay by partially denaturing the protein, subsequently reducing surface tension and unfolding the protein, thereby increasing affinity for the antibody (Wallace et al., 1998b). The addition of $H_2O_2$ oxidizes the methionine residues in the β-subunits, thereby improving the affinity of the reaction and significantly increasing the sensitivity of the assay (Knight and Muttukrishna, 1994).

Two inhibin A formats are reported below; format I involves the heating pre-treatment step and format II, essentially the same as format I, differs only with exclusion of the heating pre-treatment. Both formats are presented in detail below.

In both formats, a standard curve is generated by serially diluting purified recombinant human inhibin A in FCS. Sample concentrations are then derived from sample absorbance at 490nm in a microplate reader, with a reference wavelength of 620nm, and subtraction of blank values from the standard curve. The minimum detection of the assay is less than 5pg/mL (derived from mean ± 3 standard deviations (SD) of a plate of blanks) and there is minimal cross-reactivity with the pro-αC subunit, inhibin B or activins (Robertson et al., 1997b; Wallace et al., 1998b).

### *2.3.2.1 Inhibin A ELISA (Format I)*

1. Defrost samples, standards and controls. Vortex when thawed.
2. Defrost assay diluent (solution b).
3. If the expected inhibin level is in excess of top standard (2000pg/ml) then dilute appropriately with fetal calf serum.
4. Put 100µl of each standard and sample into a 1.5mL eppendorf.
5. Add 50µl of 6% SDS (solution c) to each standard and sample. Vortex.
6. Place all standards and samples in boiling water for 3 mins. Allow to cool (approximately 20-30 mins).
7. Add 10µl of 15% hydrogen peroxide (solution d) to each sample or standard. Vortex. Allow to stand for 30 mins.
8. Add 100µl of assay diluent to each tube.
9. Remove E4 plates from cold-room. Add 100µl sample or standard to duplicate wells on the microtitre plate.
10. Place plate on plate-shaker for 5 mins. Remove plate, cover it with a seal and incubate overnight in moist container at RT.
11. The following day, wash the plate 4 times with wash buffer (solution e) and bang dry.
12. Immediately add 50µl of the Fab R1 alkaline phosphatase conjugate (solution f) to each well. Reseal plate, shake for 5mins and incubate in moist container at RT for 2 hours.
13. Reconstitute alkaline phosphatase substrate (as per manufacturer's instructions). Mix, stand for 10 mins prior to use (if using frozen reconstituted substrate, bring to RT first before use. This step takes approximately 30-40 mins).
14. Wash plate 4 times in wash buffer and bang dry.
15. Immediately add 50µl of alkaline phosphatase substrate. Reseal the plate, shake for 5mins and incubate in moist container at RT for 2 hours.
16. Reconstitute alkaline phosphatase amplifier. Mix, stand for 10 mins prior to use (if using frozen reconstituted amplifier, bring to room-temperature first before use. This step takes approximately 30-40 mins).
17. Add 50µl of amplifier to each well. Watch as colour develops and stop with 50µl of 0.4M HCl (solution h).

18. Read plate at 490nm and 650nm using a microplate reader with dedicated software (Softmax).

#### *2.3.2.2 Inhibin A ELISA (Format II)*

The steps in this assay are identical to format I above (section 2.3.2.1), except for exclusion of step number 6 (the heating pre-treatment).

### 2.3.3 Inhibin B ELISA

Inhibin B was measured using a similar two-site ELISA to that of inhibin A. The detailed protocol, assay buffers and wash solution were essentially identical. A monoclonal antibody (C5) raised against the carboxy terminal peptide of the human inhibin $\beta_B$ subunit was used as a capture antibody and immobilised on microplates. The same second antibody (Fab R1) used in the inhibin A assay was employed. Samples and standards were also pre-treated as detailed above. The recombinant human 32k inhibin as used in the inhibin A assay was used as standard, but calibrated against recombinant human inhibin-B. Plates were read at 490nm using the same hardware as detailed for the inhibin A assay. The assay detection limit was less than 15pg/mL. The cross-reactivities of this assay for activin A, activin B, follistatin and purified human pro-αC were <0.1%, with cross-reactivity with recombinant inhibin A of 0.5% (Groome et al., 1996; Wallace et al., 2000).

### 2.3.4 Inhibin Pro-αC ELISA

Inhibin forms containing pro-αC were detected using another similar two-site ELISA (Groome et al., 1995). This assay uses a capture monoclonal antibody (INPRO) raised against the entire Pro region of the human α subunit, and an immunopurified standard calibrated against recombinant human 32k inhibin (Groome et al., 1995). Unlike the inhibin A and inhibin B assays, after diluting samples with pro-αC diluent, standards and samples were added directly to the plate without pre-assay oxidation or boiling. The same second antibody (Fab R1) to the αC subunit peptide (aa 1-32) used in the inhibin A and B assays, were employed. Recombinant forms of inhibin A, inhibin B and follistatin all cross-react less than 0.02% (Groome et al., 1995), although this antibody may cross-react with the larger dimeric inhibin isoforms containing the α-

subunit pro sequences, as demonstrated by immunoblotting studies (Groome et al., 1995). The detection limit was less than 2pg/mL.

## 2.4 VALIDATION OF THE INHIBIN ELISAs

### 2.4.1 METHODS

#### 2.4.1.1 Serum stability study

Blood samples were collected in Vacutainers by routine venepuncture from ten women in late pregnancy attending the Antenatal Clinic at Monash Medical Centre. Within 30 mins of collection, each sample was divided into two aliquots. One aliquot was centrifuged for 15 mins at room temperature (RT). The serum was then removed and placed into 15ml centrifuge tubes. The other aliquot was left unseparated at RT. Both serum and unseparated samples were incubated at RT on the benchtop for 7 days. After vortexing, a sub-aliquot (0.5ml) was removed from each serum aliquot after 0, 1, 2, 3 and 7 days incubation, placed in 2ml eppendorfs and frozen at –20°C. The unseparated samples were treated in the same way, with samples being centrifuged before frozen storage of the serum. Samples were then thawed at a later date and assayed for inhibin A (using both format I and II) and pro-αC. Inhibin B levels are too low for detection in pregnancy sera.

#### 2.4.1.2 Freeze-thaw study

***A. Serum***

Blood samples were collected, with written informed consent, from five healthy women in early pregnancy. In each case, the serum was separated into 5 aliquots. On the day of collection, the fresh sample was assayed, without freezing, for inhibin A and pro-αC. Each of the other aliquots was exposed to from one to five freeze-thaw cycles and then assayed for inhibin A (assay format I) and pro-αC.

***B. Amniotic fluid***

Similarly, five Down syndrome amniotic fluid samples, which had already been assayed for inhibin A, B and pro-αC after one freeze-thaw cycle, were subsequently assayed after exposure to a further 1-5 freeze-thaw cycles. These samples were

collected prospectively at Monash Medical Centre as a component of the prenatal diagnostic service and were stored at –20°C until assay.

### 2.4.1.3 Statistical Analysis

All results are presented as mean ± standard error of the mean (SEM). Significance was determined by two-way analysis of variance (ANOVA) for repeated measures. Statistical analyses were performed using the software package Prism (Berkeley, CA, USA). Comparative analyses was considered as significant when $P<0.05$.

## 2.4.2 RESULTS

### 2.4.2.1 Serum stability study

Figure 2.1 shows the percentage change (mean ± SEM) of inhibin A levels in the ten samples, measured by formats I and II and by method of storage. Using format II (no heating pre-treatment), mean inhibin A levels in samples stored as whole blood declined significantly by more than 50% ($p<0.01$) after 7 days, whereas levels were stable for the samples stored as serum. When format I was employed however, no significant changes in mean inhibin A levels were observed, regardless of how samples had been stored ($p=0.08$ for both formats I and II) (figure 2.1).

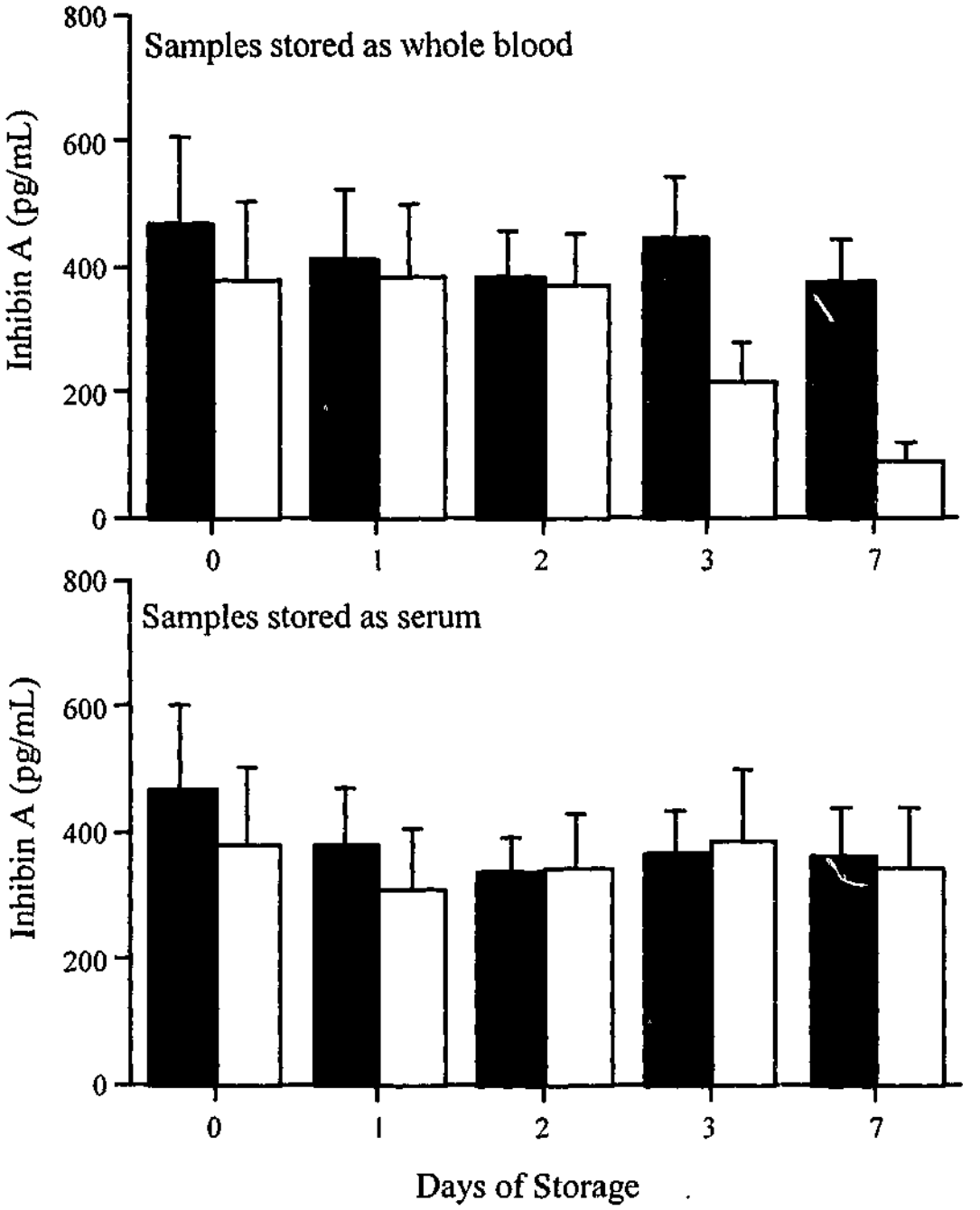



**Figure 2.1:** Changes in inhibin A concentrations (mean ± SEM) in samples from 10 pregnant women. ■ = measured using format I (heating pre-treatment); □ = measured using format II (no heating pre-treatment).

Similarly, figure 2.2 shows mean (± SEM) pro-αC levels in samples stored as whole blood declined abruptly ($p<0.01$), with a fall of 70% after 7 days. Again, levels were stable for samples stored as serum (figure 2.2).

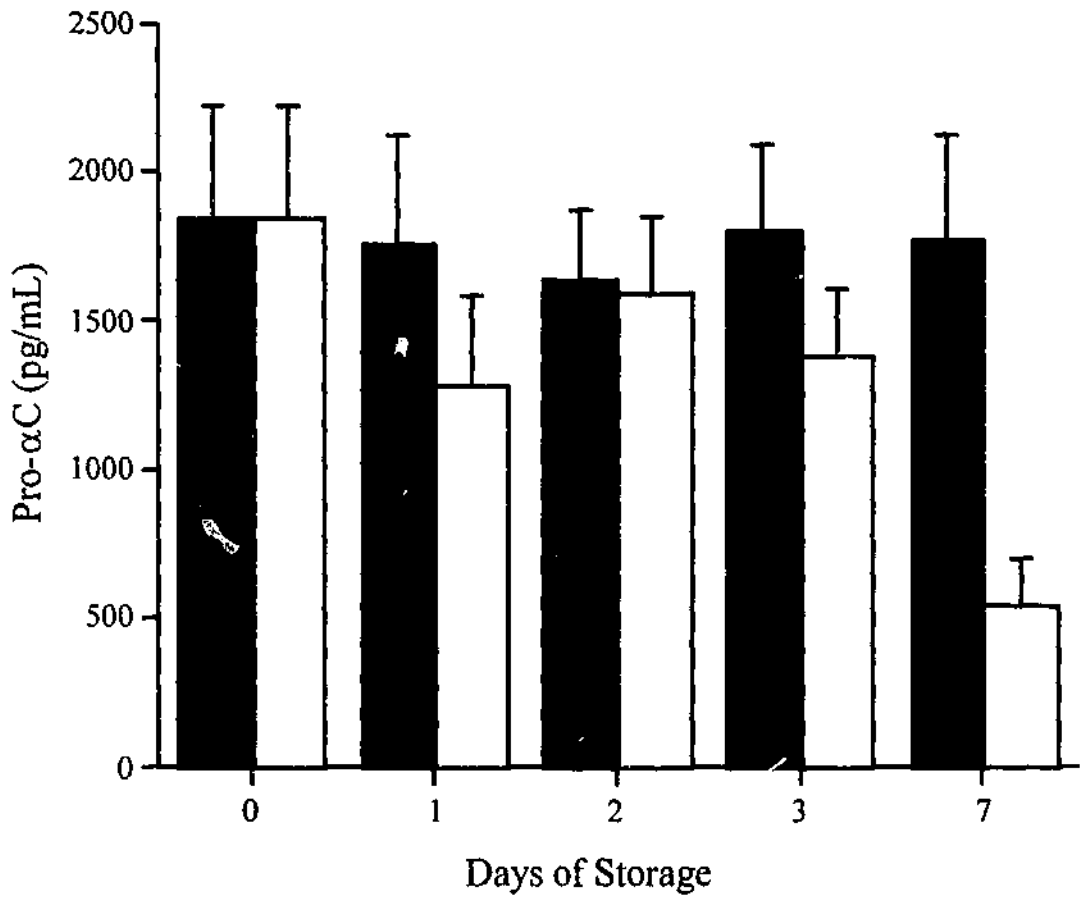



**Figure 2.2:** Changes in inhibin Pro-αC concentrations (mean ± SEM) in samples from 10 pregnant women. ■ = samples stored as serum; □ = samples stored as whole blood.

### 2.4.2.2 Freeze-thaw study

*A. Serum and amniotic fluid*

There were no significant changes in any of the inhibins measured in serum (inhibin A and pro-αC) (figure 2.3) or amniotic fluid (inhibins A, B and pro-αC; figure 2.4) with repetitive freeze-thawing of up to five cycles.

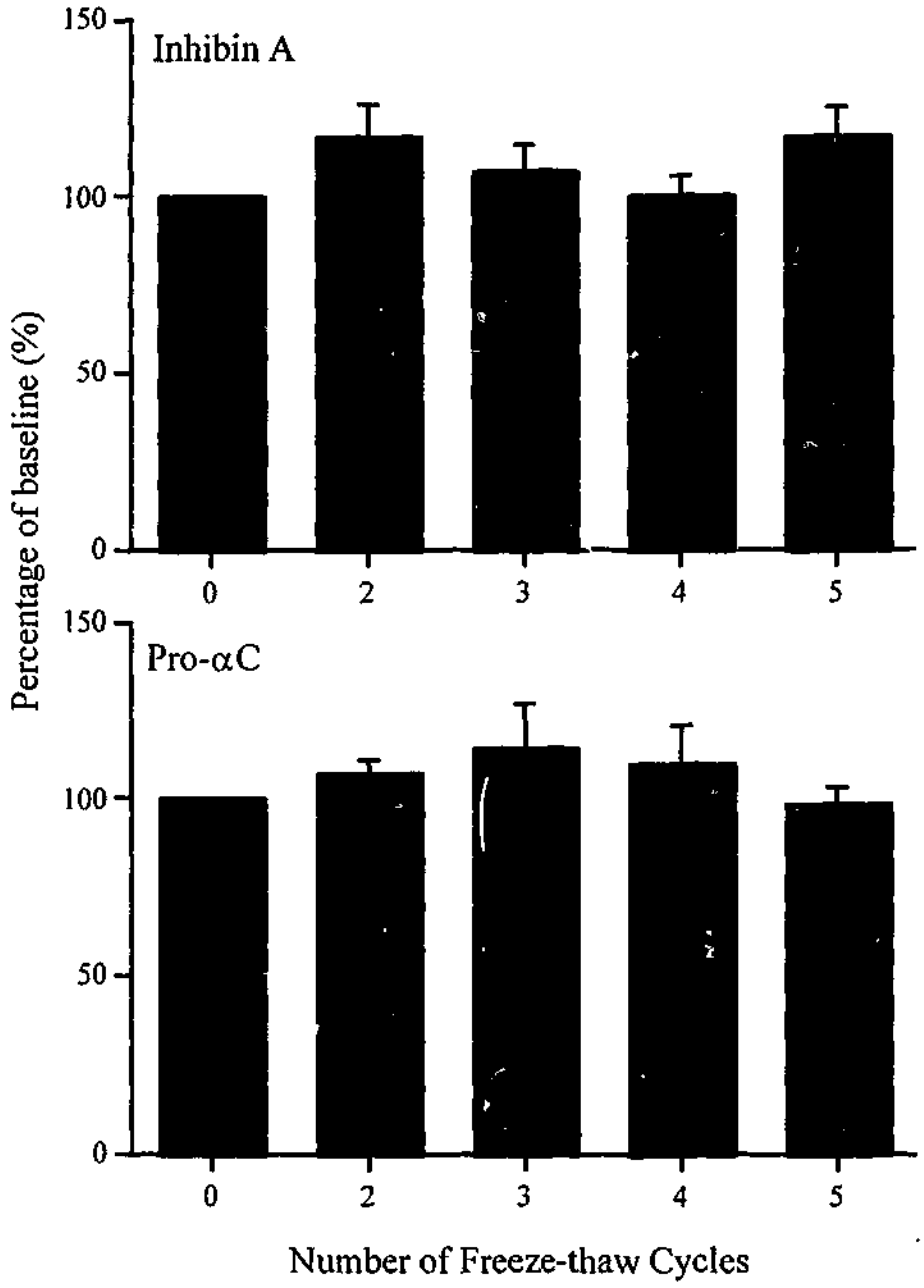



**Figure 2.3:** The percentage (%) change of inhibin A and Pro-αC concentrations (mean ± SEM) in five maternal serum samples from after 1-5 freeze-thaw cycles.

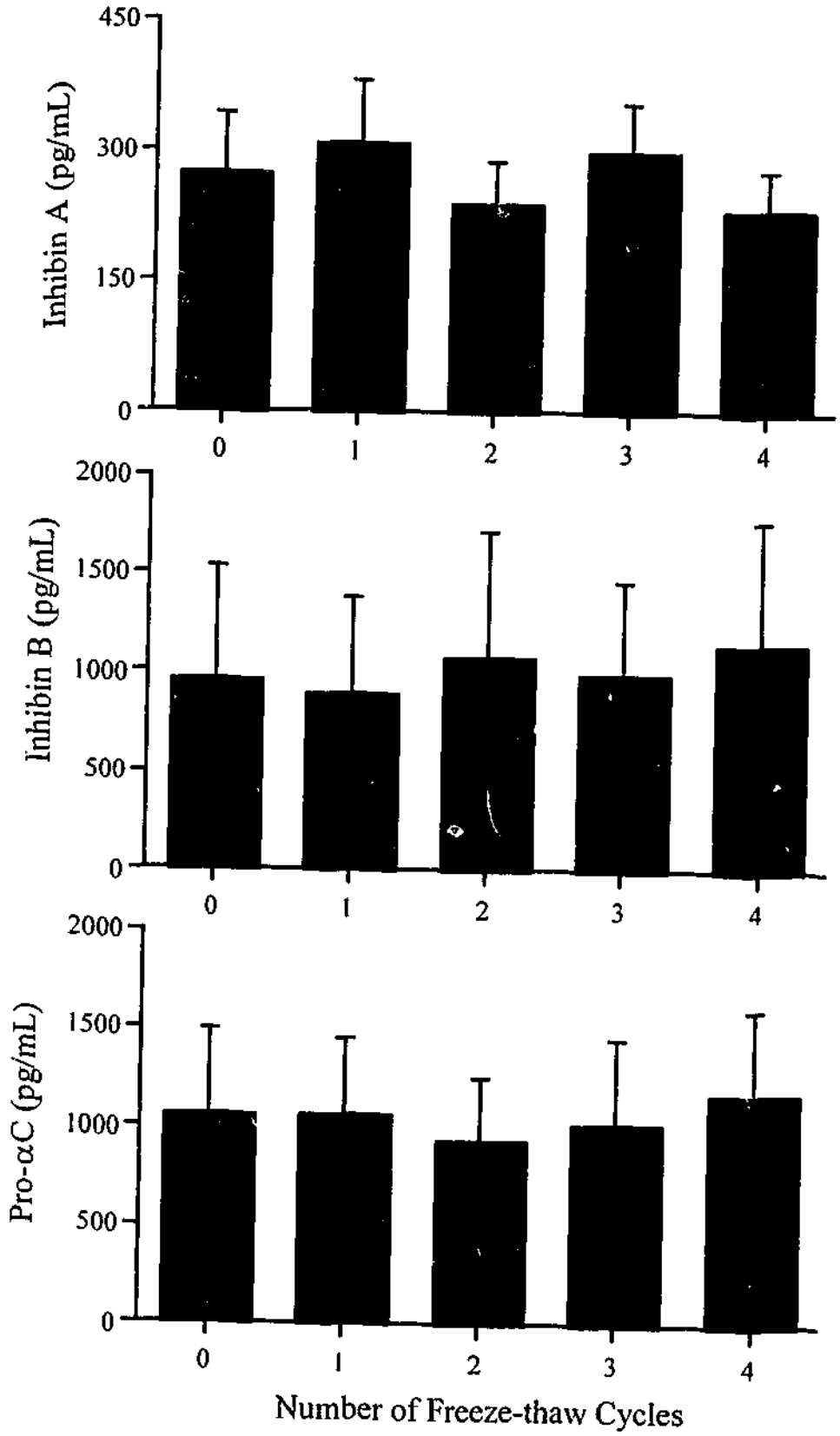



**Figure 2.4:** Changes in inhibin A, inhibin B and pro-αC concentrations (pg/mL; data is expressed as mean ± SEM) in 5 Down syndrome amniotic fluid samples after 1-4 freeze-thaw cycles.

## 2.5 CATALASE STUDY

### 2.5.1 INTRODUCTION

The previous section confirms the findings of Wallace and colleagues (1998b) that detectable inhibin A levels are significantly decreased by sample storage as whole blood. It was proposed that this effect was due to erythrocyte catalase interference with a critical oxidative step in the assay (Wallace et al., 1998b) and that the pre-assay heating inactivated catalase, therefore mitigating the decrease in inhibin levels. Catalase activity can also be inhibited, *in vivo* or *in vitro*, by 3-amino-1,2,4-triazole (AT), a specific catalase "suicide" inhibitor (Williams et al., 1985; Darr and Fridovich, 1986; Aragon et al., 1991). The studies related in the next section of this chapter therefore examine whether the addition of AT to samples prior to assay for inhibin A might be a simpler alternative to the heating step, affording the improvements in assay performance more cost-effectively.

### 2.5.2 METHODS

#### 2.5.2.1 Blood Samples

Blood samples were collected, with informed consent, from women in the late first trimester or early second trimester of pregnancy attending the antenatal clinic at Monash Medical Centre. Within an hour of collection, each sample was divided into 2 aliquots. One aliquot was immediately centrifuged and the serum subdivided into two subaliquots for either immediate storage at –20°C or for storage at room temperature for seven days prior to freezing. The second whole blood aliquot was kept unseparated at room temperature, mimicking "field conditions", and centrifuged after seven days. The resulting serum from this second aliquot was divided into 0.3ml sub-aliquots which were treated as detailed in the studies below. Unless otherwise stated, inhibin A was measured using formats I and II, detailed above.

#### 2.5.2.2 Determination of optimum AT concentration

In the experiments detailed below, 3-amino-1,2,4-trizole (Sigma Aldrich, Castle Hill, NSW, Australia) was prepared in 10mM phosphate-buffered saline (PBS) on the day of use and stored at 4°C until required. The manufacturer's product information details that reconstituted AT is stable at –20°C for several months.

To establish an optimum AT concentration for use in the inhibin A ELISA, blood samples were collected from six women and each sample was divided into two aliquots. One aliquot was separated immediately by centrifugation and the serum removed and stored at –20°C on the day of collection. This served as the 'baseline' control. Following storage for seven days at RT, the remaining whole blood aliquots were centrifuged and the serum collected into 2ml microcentrifuge tubes. Samples were then assayed for inhibin A using assay format II (no heating pre-treatment) with the addition of 10µl AT in varying concentrations (0-500mM, final concentration) immediately prior to assay.

**2.5.2.3 Effect of AT on inhibin A levels**

Blood was collected by routine venepuncture from a further ten healthy women in early pregnancy. On the day of collection, each blood sample was divided into two aliquots. One aliquot was centrifuged and the serum separated while the other was kept unseparated at RT. The serum from the first aliquot was further divided into three 0.3ml subaliquots, one of which was frozen at –20°C immediately. The remaining two serum subaliquots were kept at RT for seven days, with subsequent assay of one after addition of 10mM AT (final concentration) and the other after addition of PBS alone to act as a control. The whole blood aliquots were stored at RT for seven days prior to being centrifuged. The resulting serum was divided into two aliquots for the addition of AT or PBS as described above.

**2.5.2.4 Effect of catalase on inhibin A measurement**

To test directly the effectiveness of AT as an inhibitor of catalase in serum, bovine liver catalase (Type C10; Sigma Aldrich, Castle Hill, NSW, Australia) was prepared at varying concentrations (final concentration: 2mg/mL – 32mg/mL) in 10mM PBS. Blood was collected by routine venepuncture from a further ten healthy women in early pregnancy and a 0.3ml serum aliquot from each sample prepared on the day of collection. To each aliquot, catalase in varying concentrations, was added prior to assay for inhibin A with or without the further pre-assay addition of 10mM AT.

### 2.5.2.5 Statistical Analyses

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc, CA, USA). The concentrations of inhibin A in blood/serum after storage and incubation with AT were subjected to two-way analysis of variance (ANOVA) for repeated measures. Significance was accorded when $P<0.05$.

## 2.6 RESULTS

### 2.6.1 Determination of optimum AT concentration

Figure 2.5 shows the effect of AT on inhibin A levels. The concentrations of AT that produced optimum inhibin A levels ranged from 10-80mM. There were no significant differences in mean inhibin A levels between freshly frozen samples (baseline) and samples stored as whole blood with these concentrations of AT. Concentrations of AT below and above this were associated with lower levels of detectable inhibin A. In samples without AT, inhibin A levels were significantly decreased compared with baseline levels (ANOVA, $P=0.005$). The lowest dose of AT which was associated with optimum inhibin A levels (10mM) was subsequently used in all experiments.

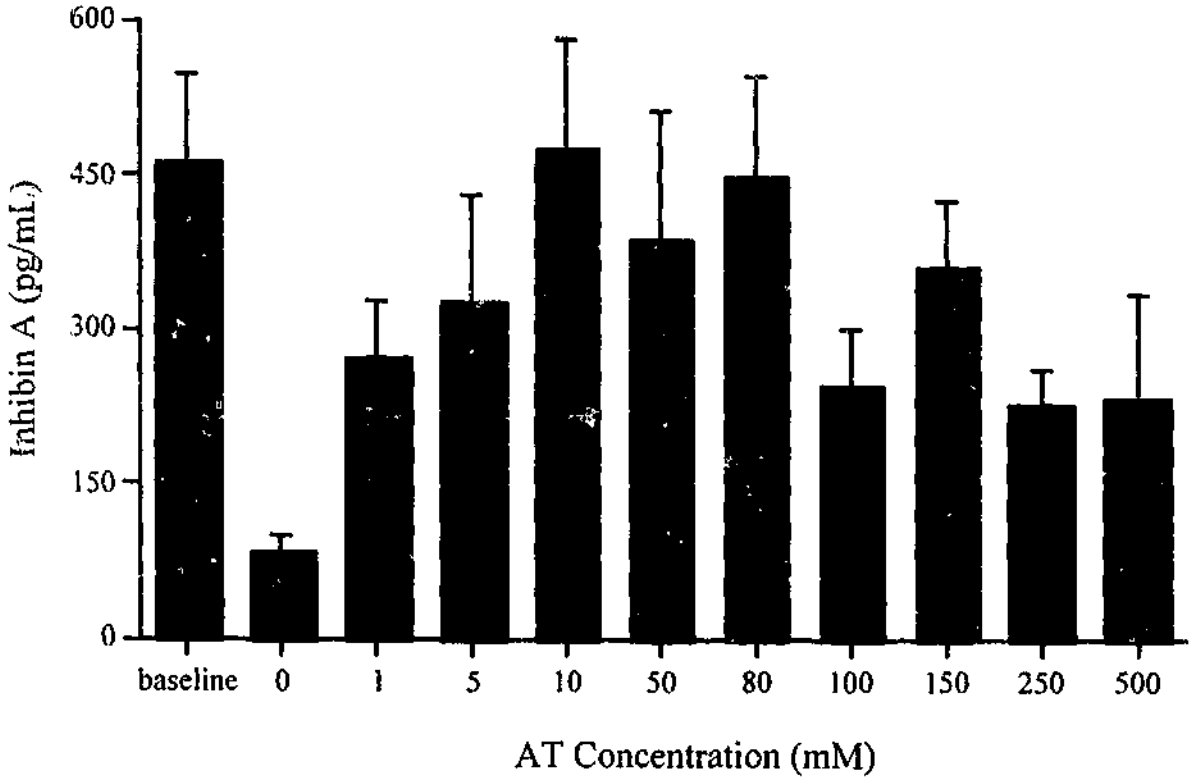



**Figure 2.5:** The effect of AT (0mM – 500mM, final concentration) on inhibin A levels (mean ± SEM) in 6 samples stored as whole blood, measured using assay format II (no heating pre-treatment).

### 2.6.2 Effect of AT on inhibin A levels

Figure 2.6 below shows the effect of method of sample storage and the assay format used, with and without the addition of 10mM AT, on the detectable inhibin A level. The mean ± SEM inhibin A level in baseline samples was 483.8 ± 124.8 and 462.6 ± 50.4 for format I and II, respectively. Using format I (pre-assay heating), no significant changes in mean inhibin A level were observed (figure 2.6), regardless of how the samples had been stored or whether AT had been added or not. When assayed using format II, the mean inhibin A level in samples stored as whole blood declined by 85% after seven days (P=0.003) whereas levels were stable for the samples stored as serum, consistent with the previous finding (figure 2.1). The decline in inhibin A in the whole blood samples was prevented with the addition of 10mM AT (462.6 ± 50.4 versus 457.2 ± 36.3; P=0.323).

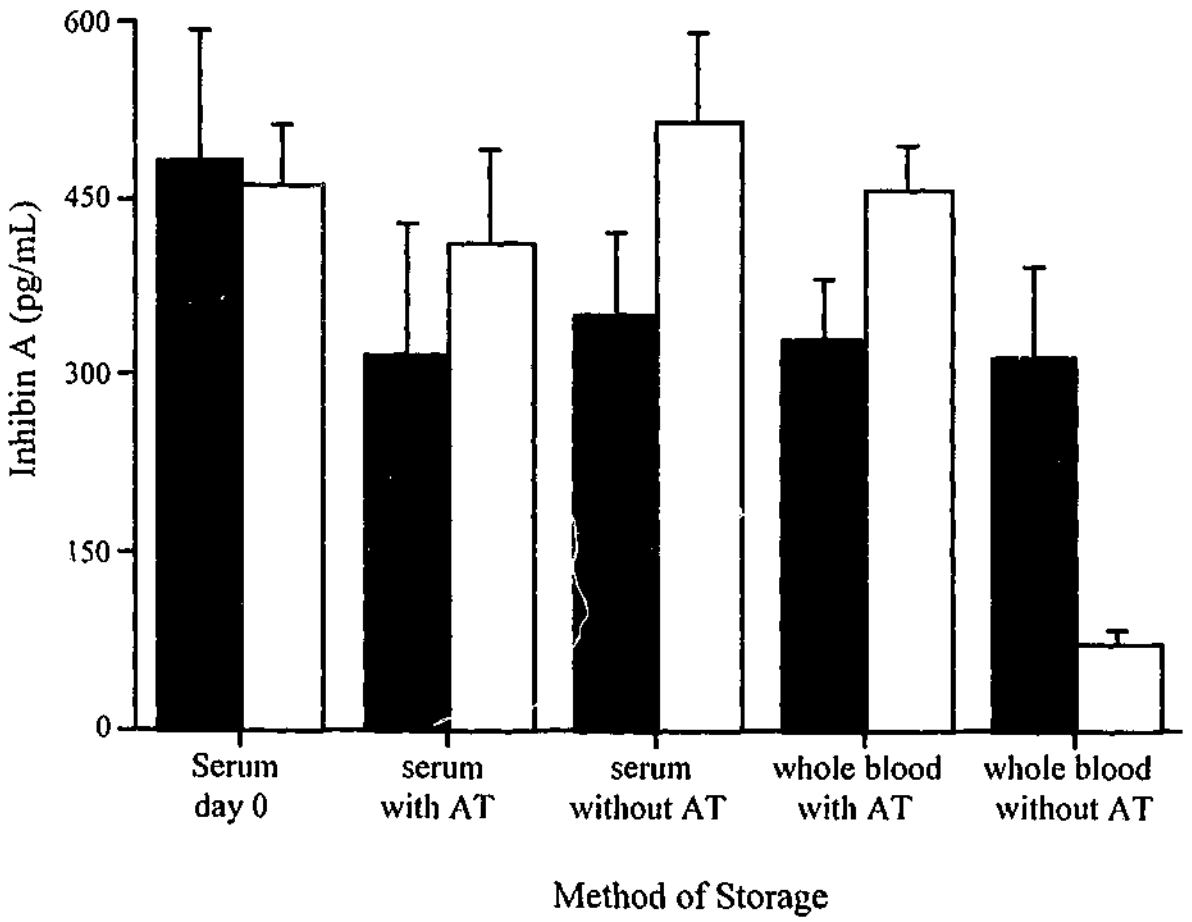



**Figure 2.6:** The effect of 10mM AT on inhibin A levels (mean ± SEM) in samples stored as serum or whole blood. ■ = samples measured using assay format I (heating pre-treatment); □ = samples measured using assay format II (no heating pre-treatment).

### 2.6.3 Effect of catalase on inhibin A measurement

The addition of catalase without AT led to a significant reduction in the inhibin A detectable (ANOVA P=0.001) which was entirely mitigated by the addition of 10mM AT (figure 2.7).

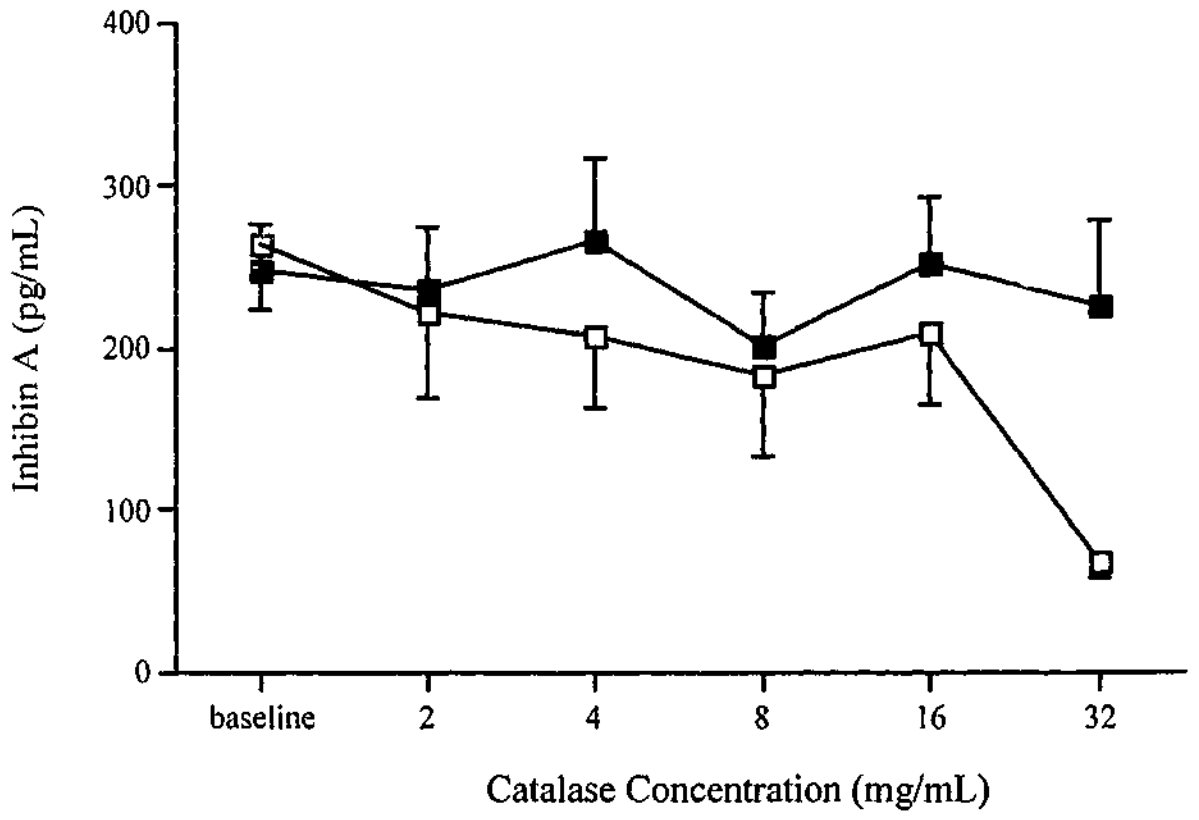



**Figure 2.7:** The effect of catalase (2mg/mL – 32mg/mL) on inhibin A levels (mean ± SEM), measured using assay format II (no heating pre-treatment), in 10 serum samples. ■ = with pre-assay addition of 10mM AT; □ = without pre-assay addition of 10mM AT.

## 2.7 DISCUSSION

This chapter describes in detail the different inhibin immunoassays (inhibin A, inhibin B and pro-αC) used in the studies reported in this thesis. The simple stability studies show that inhibins, as detected by the assays, are stable under repetitive freeze-thaw cycles to at least 5 cycles. Further, the studies show that inhibin A and pro-αC levels decline when samples were stored, at room temperature, as whole blood but not as

serum, and that when the samples were boiled prior to assay, this effect was prevented.

The changes observed with format II (no boiling) could not have been due to dissociation of the inhibin dimer, because levels were stable with format I (SDS boiling), but rather to an effect related to storage as whole blood, but not serum. This is quite different to hCG which dissociates during storage (Sancken and Bahner, 1995), an effect recently shown to be secondary to microbial action (Kardana and Cole, 1997). It has been suggested that the effect observed for inhibin is due to the release of catalase from red blood cells during storage (Wallace et al., 1998b). Catalase is thought to quench the oxidising effect of the critical hydrogen peroxide pre-treatment step, required to oxidize the epitope recognised by the E4 anti-$\beta_A$ subunit antibody thereby increasing sensitivity of the assay (Knight and Muttukrishna, 1994). Indirect supporting evidence for this suggestion was afforded by the observation that heating, which would denature the catalase, prevented the quenching. Importantly, a quenched inhibin A signal was evident in the stability study samples even without obvious haemolysis. These data therefore suggest that while the apparent inhibin A level is stable in clinical samples, this is only so when using format I.

To date, optimisation of the commercially available inhibin A ELISA (Oxford Bio-innovations, Upper Heyford, UK) for whole blood samples that had not been separated on the day of collection required heating the samples prior to assay to prevent erythrocyte catalase interference (Wallace et al., 1998b). This study confirmed that, if the sample heating step is omitted from the assay, when whole blood samples are kept unseparated for seven days, mimicking field conditions, measured inhibin A levels are significantly lower than when either assayed in a fresh sample or when the sample is separated fresh and stored as serum. It was previously shown that this effect also reduced discrimination, albeit marginally, between Down syndrome and normal pregnancies (Wallace et al., 1998b). However, results from this chapter have also shown that the effect of the sample heating, an awkward and labour-intensive step in the assay, can also be achieved by the simple addition of 3-amino-1,2,4-triazole (AT).

AT is a specific inhibitor of catalase activity both *in vitro* and *in vivo* (Heim et al., 1956; Williams et al., 1985). Following its injection in rats, AT reduces both hepatic and renal catalase activity (Heim et al., 1955) and more recently, AT inhibition of brain catalase has been confirmed in vivo by several authors (Sinet et al., 1980; Tampier et al., 1988; Aragon et al., 1989). Relevant to the use of AT in the inhibin ELISA, the inhibition of catalase by AT requires the presence of hydrogen peroxide (Tehphyl et al., 1961; Nicholls, 1962; Darr and Fridovich, 1986). During the process whereby catalase breaks down hydrogen peroxide into water and oxygen, catalase attains a high oxidative state, known as compound I, generated by interaction with the first molecule of hydrogen peroxide (Ou and Wolff, 1996). It is with compound I that the AT reacts and irreversibly inhibits catalase.

When samples were treated with increasing concentrations of bovine liver catalase, detectable inhibin A levels declined. This decline was mitigated, at all concentrations of catalase, by the addition of AT. However, it is possible that excessive haemolysis, which was not visually present in any of the samples used the experiments detailed here, may be associated with significant enough catalase leakage to overcome the standard dose of AT described here. Further studies of this possibility would be merited by laboratories that encounter a significant percentage of badly haemolysed samples and that do no use the pre-assay heating step.

In summary, this chapter has confirmed that detectable inhibin A levels are significantly decreased by sample storage as whole blood but that this effect can be mitigated by either pre-assay sample heating or by the addition of the catalase inhibitor, 3-amino-1,2,4-triazole. Clearly, these observations are important because samples collected for many prenatal screening programmes might travel for some distance and time prior to assay. Accordingly, it is suggested that the simple addition of AT to samples in the ELISA plate prior to assay for inhibin A may be a simple but valuable assay modification.

# CHAPTER THREE

## Immunoaffinity Fractionation

## Chromatography: Methodology & Validation

## 3.1 INTRODUCTION

As discussed previously (Chapter One, section 1.2), it has been recognized that inhibins exist as a number of molecular weight forms. These forms result from the $NH_2$-terminal processing of both the α- and β subunits at recognized cleavage sites or to differential glycosylation of the α-subunit (Mason et al., 1996a). In addition, various processed forms of the biologically inactive α-subunit have also been identified (Knight et al., 1989; Robertson et al., 1989; Sugino et al., 1989; Lambert-Messerlian et al., 1994). The inhibin molecular weight forms present in human follicular fluid (hFF) (Robertson et al., 1997a), serum from women during IVF cycles (Robertson et al., 1996, 1997a), postmenopausal female serum and male plasma (Robertson et al., 1995, 1997b) have been detailed previously. However, to date, the inhibin molecular weight forms in pregnancy serum have not been investigated in detail.

The purification of inhibin has proved difficult largely due to the hydrophobic nature of the protein, the lack of specific assays to monitor recoveries, and the poor yields (generally around 5%) of protein obtained. Initially, attempts to purify inhibin from follicular fluid using conventional protein purification techniques were unsuccessful (Jansen et al., 1981; Dobos et al., 1983). More successful attempts to purify inhibin from follicular fluid were aided by techniques which exploited the physicochemical properties of inhibin. These techniques included combinations of dye affinity and phenyl-sepharose chromatography, gel filtration, immunoblot, immunoaffinity chromatography, reversed-phase HPLC and SDS-PAGE (Ling et al., 1985; Rivier et al., 1985; Miyamoto et al., 1985, 1986; Robertson et al., 1987b; de Kretser et al., 1994). Earlier studies on inhibin forms in biological fluids were conducted using immunoblot analysis (Knight et al., 1989; Ireland et al., 1994; Sugino et al., 1992) or gel-permeation chromatography (Muttukrishna et al., 1995; Khalil et al., 1995). However, these two methods have limited resolution and may not readily detect relatively low levels of large or small molecular weight inhibin forms (Thirunavukarasu et al., 2001b). More recently, a more sensitive fractionation procedure, developed initially to assess the specificity of new inhibin assays (Robertson et al., 1995), was used to elucidate the molecular weight forms of inhibin present in various biological fluids (Robertson et al., 1996, 1997a). Indeed, much of

the current knowledge on inhibin molecular weight forms in humans stem from this method of chromatography.

The procedure consists of partially purifying and concentrating samples by an initial immunoaffinity method using immobilized antisera raised to the α subunit of inhibin. Samples are then fractionated according to molecular size by a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/electroelution procedure. The next few chapters in this thesis utilizes this immunoaffinity fractionation procedure, with important modifications, to identify the various inhibin molecular weight forms in the different compartments of pregnancy. The modifications were introduced to cope with the larger volumes of samples used in this study and to increase the efficacy of the preparative-PAGE procedure. This particular chapter details those methods, modifications and their validation. Where appropriate in each study thereafter, reference is made to the relevant section in this chapter for methodological details.

## 3.2 MATERIALS & METHODS

### 3.2.1 Preparations

#### *3.2.1.1 Human recombinant inhibin*

Human recombinant 31-34k inhibin A was provided by Biotech Australia P/L (Roseville, NSW, Australia) as described previously (Tierney et al., 1990).

#### *3.2.1.2 Human follicular fluid (hFF)*

hFF was collected from women undergoing gonadotrophin stimulation at the time of egg pick-up from Monash IVF (in-vitro fertilization) Clinic (Clayton, Victoria, Australia). The hFF was placed on ice within minutes of collection, centrifuged (to remove blood and debris) within ~1hr and snap frozen in solid $CO_2$/ethanol. A pool (30ml hFF/patient from five patients) was prepared for immunoaffinity purification.

#### *3.2.1.3 IVF serum*

Sera from women undergoing gonadotropin stimulation as part of an IVF program (designated as IVF serum) were also obtained from Monash IVF (Clayton, Victoria, Australia). The sera had been collected as part of the hormonal tracking required

during stimulated cycles. In each case, the samples were separated and frozen within hours of collection. Serum samples from women with serum oestradiol values great than 2 nmoles/L were combined to give a serum pool (100mL) for use in the inhibin immunopurifica[illegible]

#### *3.2.1.4 Luteal phase serum*

Blood was collected in serum-gel tubes from 5 women (aged 28-37 years) during the luteal phase (after day 21) of a regular 28 day cycle. Bloods were centrifuged within 2h and the serum stored at –20°C. None of the women were taking hormonal contraception.

#### *3.2.1.5 Maternal serum*

Maternal serum samples were collected from women attending the Antenatal Clinic, Monash Medical Centre (1998-1999) at various stages of pregnancy. The women were all healthy with singleton pregnancies. Bloods were collected in plain serum-gel tubes (Greiner Labortechnik, Kremsmunster, Austria), stored at 4°C and centrifuged within 48 h of collection. The serum was then stored frozen at –20°C. Five pregnancy serum pools were prepared from equal aliquots of serum from >20 women.

### 3.2.2 Solutions/Buffers

#### *3.2.2.1 Immunoaffinity chromatography solutions*

- Phenylmethylsulphonylfluoride (pMSF) (35mg/mL), made up in acetone.
- Guanidine hydrochloride (GnHCl) (6M), made up in MQ water.
- 0.2M phosphate buffer, 20mM ethylenediaminetetraacetic acid (EDTA), pH 7.4.
- 0.1M phosphate buffer, 10mM EDTA, pH 7.4.

#### *3.2.2.2 Reversed phase high performance liquid chromatography (RP-HPLC) solutions*

- 100% Acetonitrile (AcN)
- 5% AcN, 0.1% Trifluoroacetic acid (TFA)
- 60% AcN, 0.1% TFA.

#### *3.2.2.3 Preparative Polyacrylamide Gel buffers*

- 10% SDS
- Ammonium Persulphate (APS): 100mg/mL in MQ water.
- Separating buffer: 36.33g Tris base, 0.3g SDS, made up to 100ml, pH 8.45.
- Separating gel (10%): Add 16.8ml 30% Acrylamide and 16.25ml separating buffer to 10.5ml MQ water. Degas for 25 minutes. Add 195ul APS, 20ul TEMED and 6.5ml glycerol.
- Stacking gel: Add 1ml 30% Acrylamide and 3.25ml separating buffer to 8.2ml MQ water. Degas for 5 minutes. Add 150ul APS and 15ul TEMED.
- Anode buffer: 6g Tris base, 8.96g Tricine, 5ml 10% SDS, made up to 500ml with MQ water.
- Cathode buffer: 48.44g Tris base, made up to 2L with MQ water, pH 8.9.
- Loading buffer: 1M Tris base, 0.1% Tween-40, 2mM pMSF, bromophenol blue, pH 8.0.

#### *3.2.2.4 Electroelution*

- Elution buffer in collection vessels: 0.3M Tris, 0.3M HEPES, 0.05% SDS, pH 7.8, 5% bovine serum albumin (bSA).
- Elution additives: 2% $NaN_3$, 3.5g NaCl, 12ml 5% bSA, made up to 100ml.
- Electroelution buffer used in assays: 1:7 dilution of elution buffer in collection vessels: elution additives.

### 3.2.3 Fractionation Procedure

#### *3.2.3.1 Antisera*

Antiserum #41 used in the immunoaffinity purification was obtained from sheep immunized against the fusion protein of bovine inhibin αC subunit (amino acids 165-300) purified by recombinant DNA methods (Forage et al., 1987).

#### *3.2.3.2 Immunoaffinity chromatography*

The IgG fraction of antiserum #41, as obtained by caprylic acid precipitation (McKinney and Parkinson, 1987) was coupled (~10mg protein/ml gel, 48 h at 4C) by an N-alkyl carbamate linkage to a gel matrix (Reacti-Gel 6X, Pierce, Rockford, IL,

USA). The coupled gel (5-10ml) was initially washed in 6M GnHCl followed by 10 bed volumes of 0.1M phophate buffer, pH 7.0. In a typical fractionation run, samples (30ml) were rapidly thawed in the presence of 20ml 0.2M phosphate buffer, pH 7.4, containing 20mM EDTA, 2mM pMSF, 1.67% Triton X-100 (Merck, Kilsyth, Victoria, Australia). The mixture (50ml) was added to 60ml gel-immobilised IgG cut of antiserum, raised in sheep against a human inhibin αC subunit fusion protein (Robertson et al., 1996) and incubated at 4°C overnight on a rotating wheel. The gel was added to an empty 10ml column (PD-10, Pharmacia, Uppsala, Sweden). An aliquot of the supernatant was set aside for assessment of recoveries and the remainder of the supernatant discarded. The gel was washed with 0.1M phosphate buffer, pH 7.4, 10mM EDTA and brought to room temperature before elution with 6M GnHCl.

### *3.2.3.3 RP-HPLC*

The GnHCl fraction (30ml) was diluted with 30ml water, divided into 10ml lots and fractionated on 6 disposable C18 reverse phase cartridges (Vydac Bio-Select extraction columns, Separations Group, Hesperia CA) which had been prewashed with 100% AcN (3ml) followed by 5% AcN/0.1% TFA (3ml). The columns were eluted with 60% AcN/0.1% TFA (3ml) (solution g) and the 6 eluates pooled (18ml), SDS (final concentration 0.5% v/v) added and lyophilised for 5 days to ensure removal of all TFA. Aliquots for assessment of recoveries were lyophilized separately in bSA.

### *3.2.3.4 Prep-PAGE and electroelution*

The lyophilized sample was dissolved in 1200µl 1M TRIS pH 8.0, 2mM pMSF, 0.1% Tween-40, bromophenol blue (assisted by sonication), and applied to a 10% polyacrylamide gel (20 x 20cm gels) in Tris/Tricine/SDS buffers (Schagger & Jagow, 1987). Coloured molecular wt standards (6.5-205kDa, Sigma Aldrich, St Louis, Missouri) were applied either with the sample or in an outside lane. The gel was run overnight at 35mA until the 13.5k mol wt standard reached the bottom of the gel. At completion of the run, the polarity was reversed for 1 min.

Pilot experiments undertaken showed that replacing glycine with tricine increased the recoveries (Table 3.1) by allowing the small polypeptides, caught behind the broad

band of SDS micelles that forms behind the leading-ion front, to resolve into discrete bands. This, coupled with the addition of Triton X100 to the samples prior to treatment, which prevented sticking of the protein to the gel surface, greatly increased recoveries (by >30% for inhibin A and >10% for Pro-αC).

**Table 3.1:** Recoveries (%) of inhibin A and pro-αC at the Prep-PAGE step using different buffers.

| Sample | Buffer | %PAGE | Triton | Recoveries (%) | |
|---|---|---|---|---|---|
| | | | | Inhibin A | Pro-αC |
| Pregnancy Serum | Tris-Glycine | 10 | - | 32.5 | 53.1 |
| Pregnancy Serum | Tris-Tricine | 10 | - | 62.2 | 61.1 |
| Pregnancy Serum | Tris-Tricine | 10 | + | 78.3 | 69.2 |

The samples were electroeluted from the gel using an elution device (Whole Gel Eluter, Biorad, Hercules, CA). Advantages to using this device included a reduction in the amount of time preparations were kept at room temperature, omission of gel staining to locate protein bands and elimination of human error associated with excising bands out of multiple gels for subsequent electroelution (Good et al., 1995).

Preliminary experiments were undertaken to optimize the electroelution procedure. To maximize recoveries, the elution process was continued for longer (150 min) than recommended requiring an increase in buffer strength (0.27M TRIS/0.3M HEPES pH 8.0, 0.05% SDS) and the inclusion of 0.1% bSA in the collection vessel to prevent sticking of the eluted protein to the eluter surfaces. An increase in buffer strength was needed as at higher voltages the pH declined rapidly as the buffering capacity of the buffer was exhausted, heat was generated and inhibin recoveries decreased.

The electroelution process was undertaken at 250mA and continued until the voltage increased to 35V. Thirty fractions (3-4ml) were collected into weighed tubes containing 500µl 0.75% NaN3, 0.8M NaCl, 5% bSA. The recovered fractions were

immediately assayed for inhibin A and pro-αC by ELISA as described in section 3.2.4.

### 3.2.4 Assays

#### *3.2.4.1 Inhibin A ($\alpha\beta_A$) ELISA, Inhibin B ($\alpha\beta_B$) and Pro-αC*

The assay procedures detailed in Chapter Two (section 2.3) were modified to measure inhibin A, inhibin B and pro-αC in electroelution buffer. Sample (100µl) in electroelution buffer, inhibin buffer (100µl (Groome et al., 1994) or standard (100µl in buffer) followed by hydrogen peroxide (20µl, 30%) were incubated overnight at room temperature. To ensure that the elution buffer did not interfere with the assay, the absorbance of standards made up in fetal calf serum were compared with the absorbance of the same set of standards made up in elution buffer. No differences were found between the two (Figure 3.1). Otherwise, the assay protocols remained unchanged and are detailed in Chapter 2 (Section 2.3). All assays were performed within 24h of an elution.

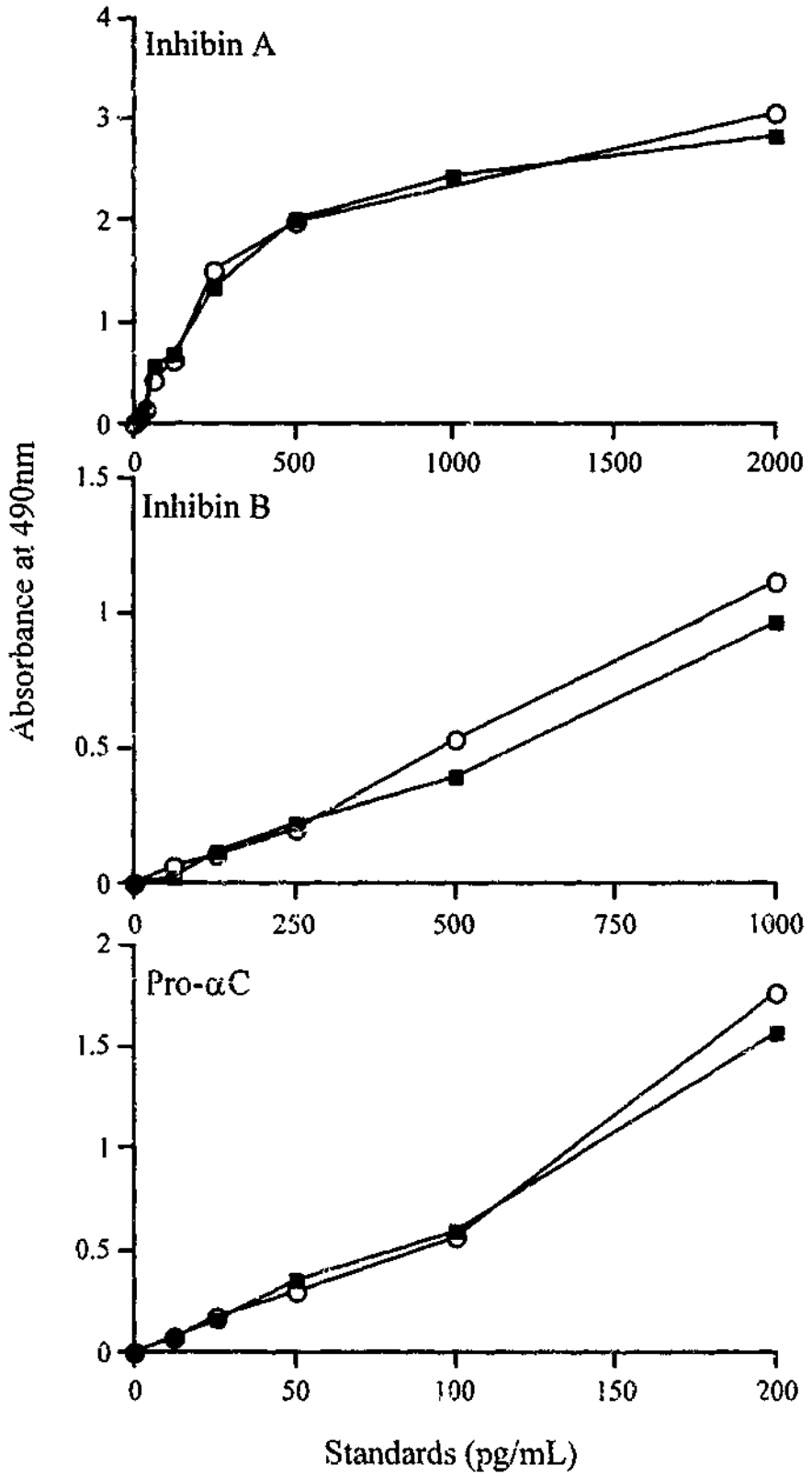



**Figure 3.1:** The absorbance of inhibin A, inhibin B and pro-αC standards in fetal calf serum (FCS ■) or electroelution buffer (○).

### 3.2.5 Data Analysis

The mol wts of the eluted Prep-PAGE fractions were calculated using protein standard markers which bracketed the 20-90 mol wt range of interest (soybean trypsin inhibiter

23.5k, bovine carbonic anhydrase 53k and bSA 82k, Sigma, Castle Hill, NSW). Regression analysis (fraction number vs log mol wt) of these 3 markers gave correlation coefficients >0.995. The resulting line of best fit was used to determine the mol wts of the eluted fractions.

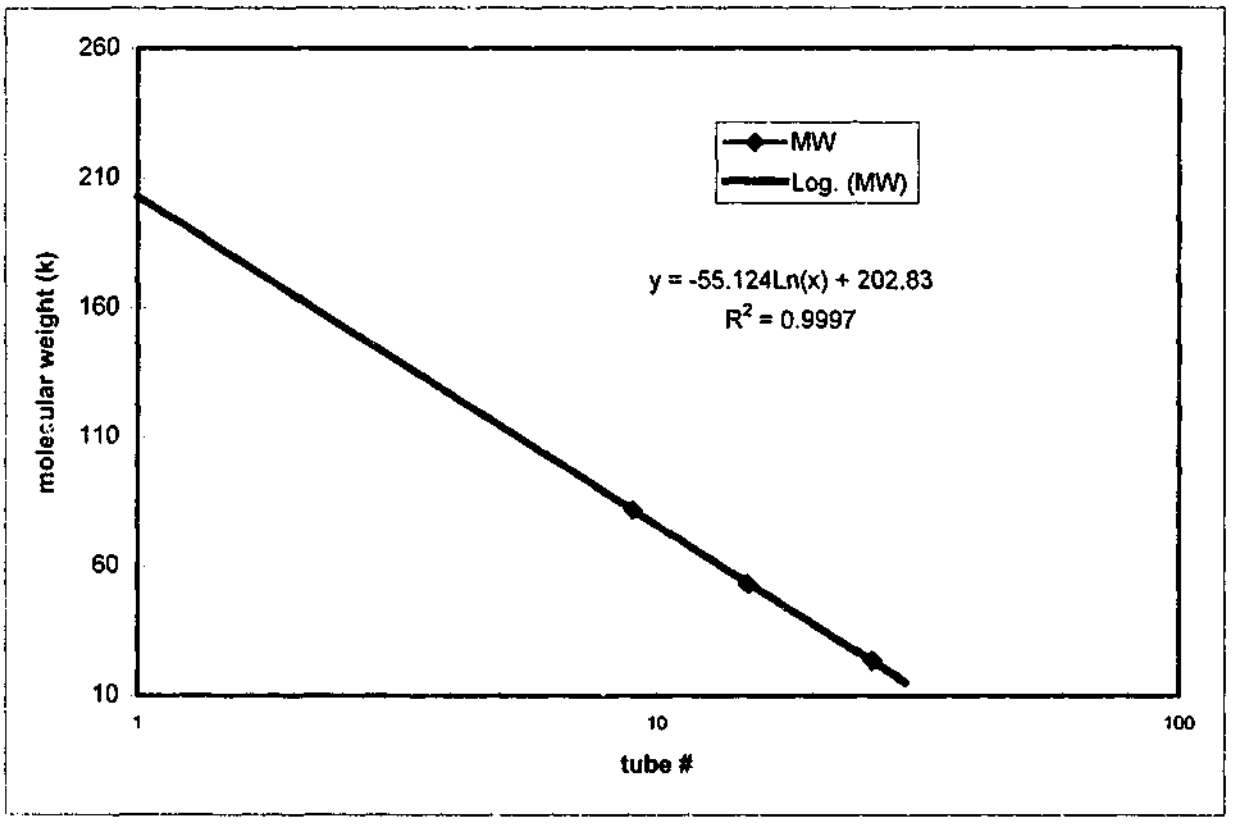



**Figure 3.2**: A typical example of the line of best fit obtained when determining the molecular weights of the eluted fractions using regression analysis of three protein standard markers (soybean trypsin inhibitor 23.5k, bovine carbonic anhydrase 53k and bSA 82k).

As a measure of precision, the mol wt determinations of the above protein standard markers when measured against the line of best fit were within 5% of their stated value. The profiles of immunoactivity were then assessed for clear evidence of peaks. Shoulders were not considered and only major peaks (>10% of recovered activity) were considered. The mol wt values of peak tubes of immunoactivity from multiple runs of a pregnancy serum pool were combined (mean ± SD) and from their distribution, activity regions were defined (eg. figure 3.5).

## 3.3 RESULTS

### 3.3.1 Validation of the Fractionation Procedure

A number of studies were undertaken to validate the modified fractionation procedure.

#### *3.3.1.1 Control*

Recombinant human 31-34k inhibin A was passed through the fractionation procedure as an appropriate control. As seen in figure 3.3, low to non-detectable inhibin immunoactivity was measured in this sample while a single 31.4k peak of inhibin A immunoactivity was recovered, representing monoglycosylated inhibin A.

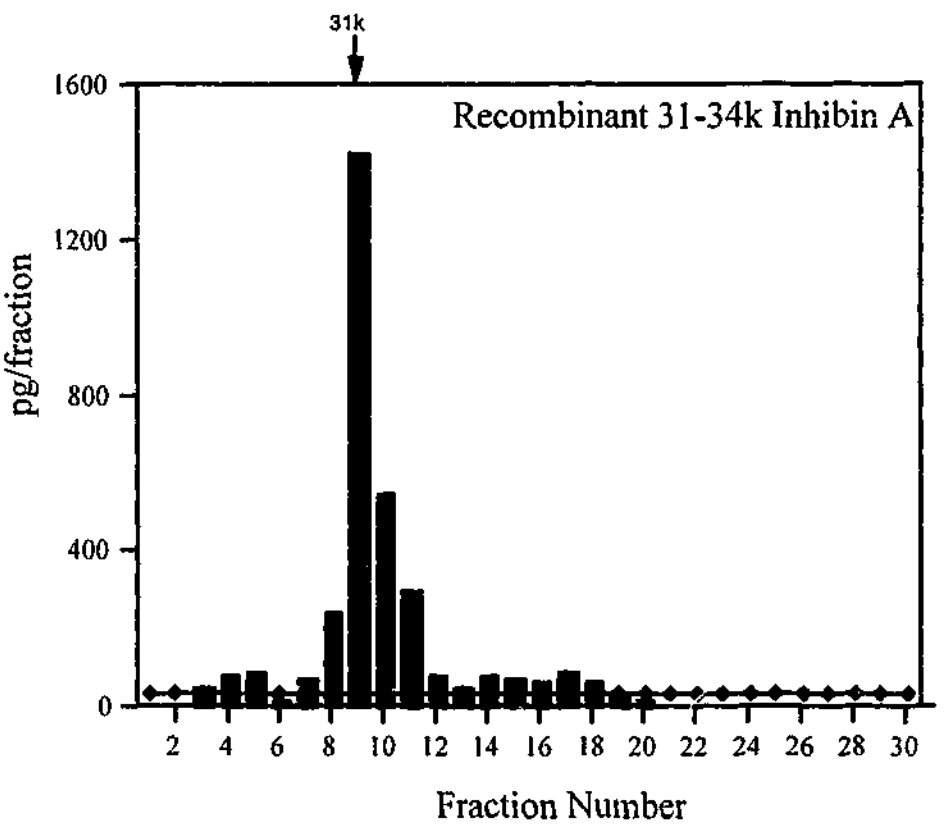



**Figure 3.3**: Molecular weight distribution of inhibin A immunoactivity in a recombinant human inhibin preparation fractionated through a combined immunoaffinity/prep SDS-PAGE procedure. The horizontal line refers to the level of sensitivity of the assay.

#### *3.3.1.2 Comparisons with previous profiles*

The mol wt profiles of inhibin A and pro-αC immunoactivity in IVF serum, a pregnancy serum pool and a luteal phase serum pool were fractionated by the new

procedure and the IVF profile compared with that obtained previously using an established immunoaffinity fractionation method (Robertson et al., 1996, 1997b). The fractionation data is presented in Figure 3.4 and the comparisons of the molecular weights of peak activities with those obtained previously are presented in Table 3.2.

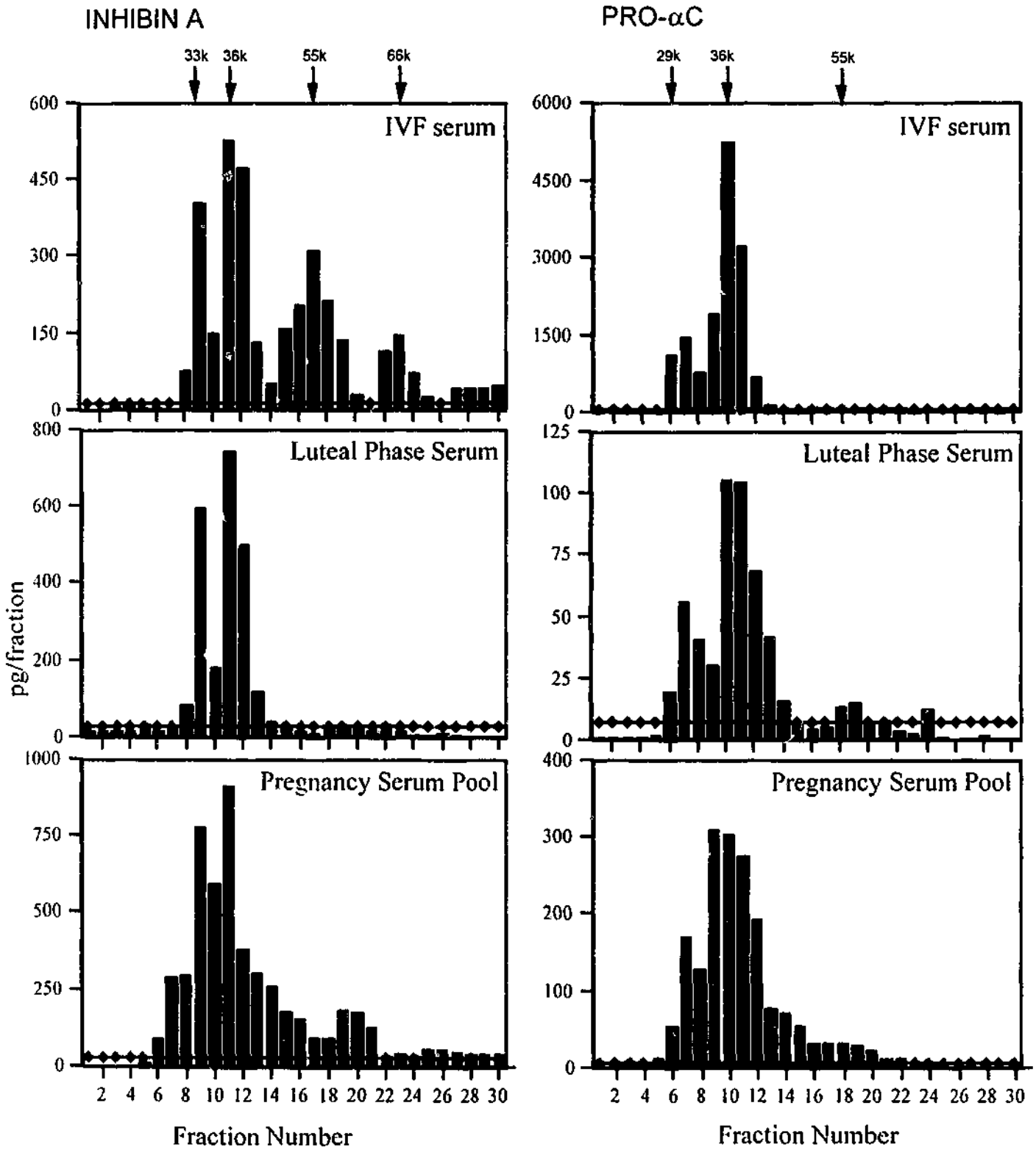



**Figure 3.4:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in serum pools fractionated through a combined immunoaffinity/prep SDS-PAGE procedure. The horizontal lines refer to the level of sensitivity of the assays.

Similar patterns of immunoactivity were noted with those published previously for IVF serum (Robertson et al., 1997b) (table 3.2), with the various mol wt forms identified consistent with partially processed and processed forms of inhibin A and inhibin α subunit containing the pro-αC fragments. The mol wt pattern of inhibin A and pro-αC in a pregnancy- and luteal phase serum pool show primarily the mature 30-36k inhibin A and 25-36k pro-αC forms (figure 3.4) with differences in mol wt within inhibin forms attributable to glycosylation differences of the α subunit.

**Table 3.2:** Comparisons of the peak molecular weight forms of inhibin A and pro-αC in IVF serum obtained in this study with that obtained by Robertson et al (1997b). (* and ** represent mono- and di-glycosylated forms).

| **Inhibin A** | | |
|---|---|---|
| **This Study** | **Robertson et al (1997)** | **Likely Form** |
| 33k | 33k | $\alpha$-$\beta_A$* |
| 36k | 36k | $\alpha$-$\beta_A$** |
| 55k | 55k | Pro-αN-αC/β |
| 66k | 66k | αC/Proβ-$\beta_A$ |

| **Pro-αC** | | |
|---|---|---|
| **This Study** | **Robertson et al (1997)** | **Likely Form** |
| 29k | 29k | Pro-αC* |
| 36k | 36k | Pro-αC** |

### *3.3.1.3 Reproducibility*

As a measure of the reproducibility and precision of the fractionation procedure, 5 replicate immunoaffinity/Prep-PAGE/electroelution runs of the same pregnancy pool were fractionated at various stages throughout the study. In order to compare between chromatograms, the inhibin A and pro-αC patterns were divided into regions based on mol wts of the peak regions and the immunoactivity values calculated as a percentage of recovered activity (figure 3.5). The combined analysis of data from the 5 runs

showed a high reproducibility with CV of recovered activity for the various mol wt regions ranging from 10-20% (figure 3.5). The profile of activity showed a clear distinction between peaks detected.

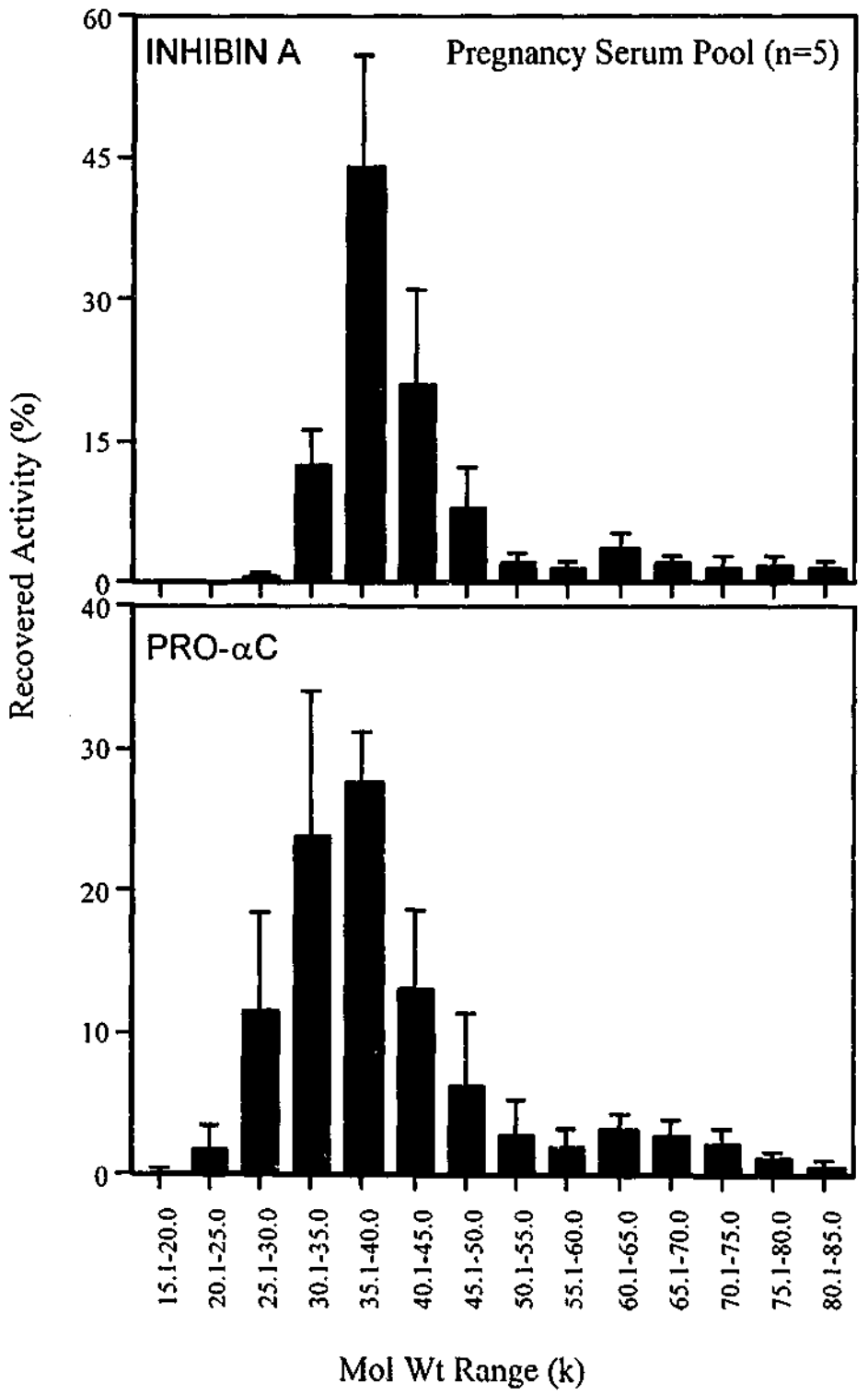



**Figure 3.5:** Mean ± SD % recovered inhibin A and pro-αC immunoactivities in a pregnancy serum pool fractionated on 5 separate occasions through the combined immunoaffinity/preparative PAGE procedure. The profile is divided up into mol wt regions for averaging purposes.

The CV of mol wts calculated for the peak tubes of immunoactivity for the 5 runs was <10% (table 3.3).

**Table 3.3:** A pregnancy serum reference pool was repeatedly fractionated (n=5) to obtain an assessment of the reproducibility of the fractionation procedure.
(* and ** represent mono- and di-glycosylated forms).

| Range (k) | Inhibin A | Likely Form | Pro-αC | Likely Form |
|---|---|---|---|---|
| 25.1-30.0 | | | 27.3 ± 1.6 (n=5) | Pro-αC* |
| 30.1-35.0 | 31.8 ± 0.8 (n=5) | $\alpha$-$\beta_A$* | 32.3 ± 0.6 (n=3) | Pro-αC** |
| 35.1-40.0 | 36.4 ± 0.8 (n=5) | $\alpha$-$\beta_A$** | 36.1 ± 0.6 (n=3) | |
| 60.1-65.0 | 61.1 ± 0.4 (n=5) | Pro.αN.αC- $\beta_A$ | 62.4 ± 2.5 (n=5) | Pro.αN.αC-β |

### *3.3.1.4 Assessment of recoveries*

Overall, 36.3 ± 12.4% inhibin A immunoactivity was recovered while 28.4 ± 10.8% pro-αC immunoactivity was recovered in combined immunoaffinity/prep-PAGE procedure. Recoveries at the RP-HPLC step were 73.8 ± 16.4% and 60.3 ± 17.8% for inhibin A and pro-αC, respectively, with 50.5 ± 20.2% and 42.3 ± 19.8% at the prep-PAGE step.

### *3.3.1.5 Assessment of immunoaffinity procedure*

To establish if inhibin underwent cleavage during the immunoaffinity procedure, hFF was fractionated either directly by prep-PAGE or by mixing with inhibin-free serum (obtained by immunoabsorption of normal non-pregnant female serum) and fractionated through the immunoaffinity/prep-PAGE procedure. Highly comparable patterns of inhibin immunoactivity were obtained by the two methods (figure 3.6), consistent with no processing during this step. Furthermore, the peaks of hFF immunoactivity in this study (33k, 36k, 55k, 66k and 97k inhibin A and 29k, 36k and

55k pro-αC molecular weight forms) (figure 3.6) matched those obtained by Robertson et al (1997a,b).

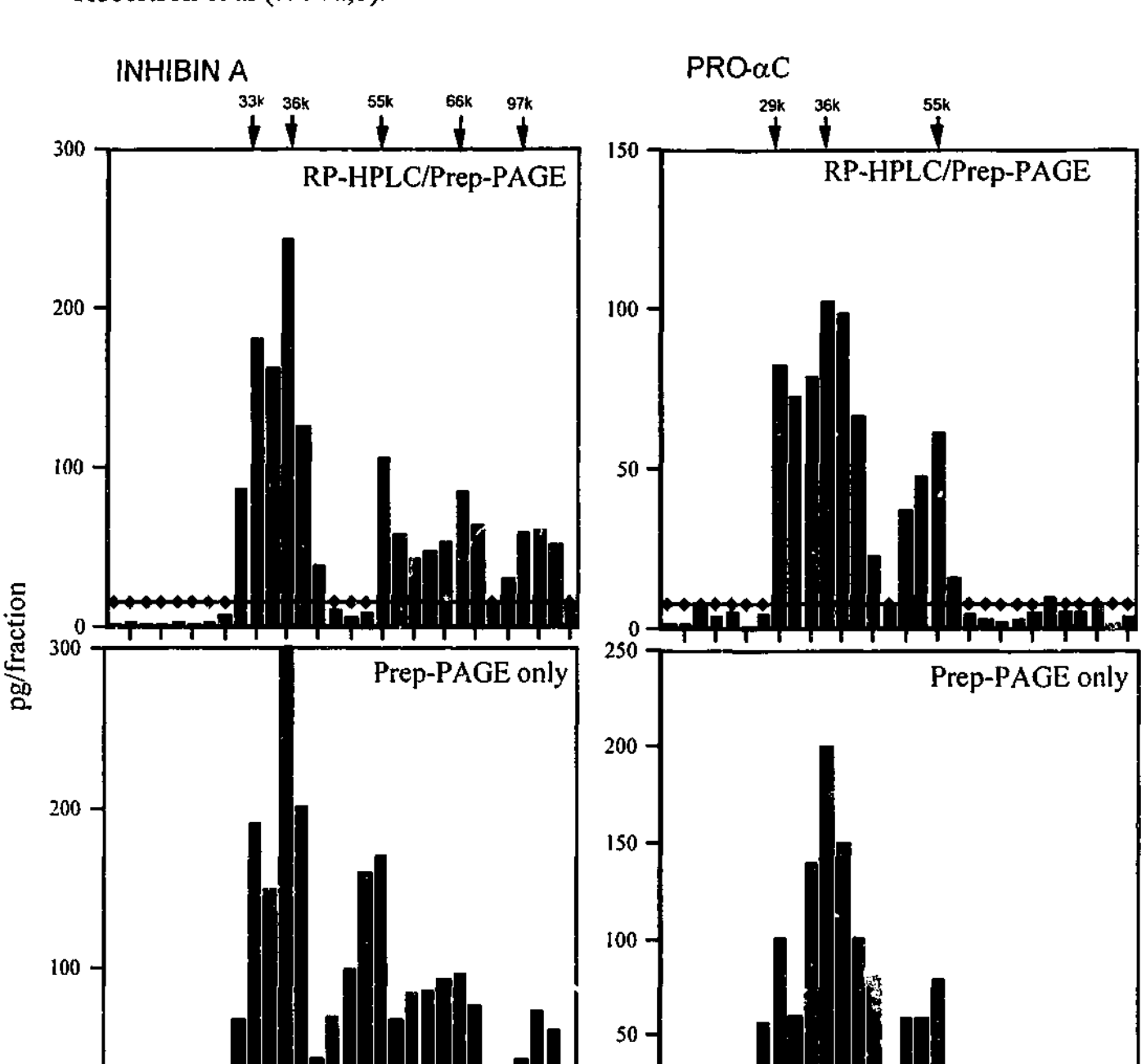



**Figure 3.6:** Inhibin A and pro-αC molecular weight profiles of human follicular fluid (hFF) fractionated either directly by prep-PAGE or by mixing with inhibin-free serum and fractionated through the immunoaffinity/prep-PAGE procedure. The horizontal lines refer to the level of sensitivity of the assays.

## 3.4 DISCUSSION

The immunoaffinity procedure detailed here was introduced to quantitatively isolate and concentrate inhibin from large volumes of serum, so required as inhibin circulates at low levels (pg per mL concentrations) in pregnancy serum. The overall recovery of inhibin activity throughout the various purification steps ranged from 28-36% with recoveries of 60-73% in the RP-HPLC step and 42-55% at the Prep-PAGE/electroelution step. This is better than recoveries obtained previously with the earlier fractionation method (Robertson et al., 1995), where recoveries at the RP-HPLC step were 50-60% and ~30% at the prep-PAGE step. The increased recoveries in this study were due to:

a) the addition of Triton-X100 to the samples prior to treatment, which prevented sticking of the protein to the gel surface,
b) replacing glycine buffer with tricine, which allowed small polypeptides, caught behind the broad band of SDS micelles that forms behind the leading-ion front, to resolve into discrete bands, and
c) an increase in the elution process time (with associated increased buffer strength to maintain sample buffering capacity in the face of higher voltages).

These three modifications, at the prep-PAGE/electroelution level, resulted in a higher resolving capacity. Although overall recoveries were still reduced, these results are not attributed to differential losses of particular inhibin forms at particular steps as Robertson and colleagues (1995) have observed non-detectable inhibin immunoactivity in immunodepleted serum, indicating that >95% of the inhibin was bound to the immunosupport. Since no modifications were made to the RP-HPLC step in this study, with each step essentially identical to that reported by Robertson and colleagues (1995), it is believed that no significant losses of inhibin occurred at the RP-HPLC step. Similarly, the 'losses' at the Prep-PAGE step have been previously shown to be similar for both high (40-200k) and low (25-40k) molecular weight forms (Robertson et al., 1995). Therefore, the low observed overall recoveries are attributed to nonspecific/irreversible binding to the various supports and to the difficulty in obtaining individual quantitative recoveries in the electroelution step due to limitations in the methodology.

The RP-HPLC procedure was used as a bulk isolation step to remove the GnHCl in samples and has been shown to be non-selective (Robertson et al., 1995). The use of a protease inhibitor (pMSF) in conjunction with the use of strong dissociants (GnHCl, SDS) and low pH ensured that cleavage/protease activity in serum was minimized and that the interference of binding proteins was negated (Robertson et al., 1995). Studies by McLachlan et al (1986b) showed that the conversion of bovine 58k inhibin to 31k inhibin was promoted by human serum, but that this conversion was inhibited separately by pMSF. As observed from the data presented in this chapter, there is little evidence of an increase in lower molecular weight forms in any of the biological fluids fractionated, suggesting that processing/metabolism during the fractionation procedure was not marked. Furthermore, when hFF was fractionated either directly by prep-PAGE or by mixing with inhibin-free serum and fractionated through the immunoaffinity/prep-PAGE procedure, highly comparable patterns of inhibin immunoactivity were obtained by the two methods, consistent with no processing during the immunoaffinity fractionation procedure.

Importantly, the nature of the forms in the biological fluids in this study matches those previously identified or isolated - from human follicular fluid and IVF serum by other investigators (Robertson et al., 1995, 1996, 1997b) and from recombinant human inhibin by Tierney et al (1990) - or those anticipated from the amino acid sequence of the inhibin α- and $\beta_A$-precursor proteins (Stewart et al., 1986; Mason et al., 1986). As detailed in Chapter One (section 1.2), the α subunit, either in the free form or as an αβ subunit dimer, is cleaved from a 65k precursor (consisting of three regions, pro (aa 1-18), αN (aa 19-248) and αC(aa 249-366), to form pro-αC and αN, or αN-αC. Similarly, the β subunit precursor is cleaved to form the proβ and β subunits (Vale et al., 1990; Burger 1992). In addition, based on studies by Tierney et al (1990), the αC subunit can be either mono (α*) or diglycosylated (α**), resulting in inhibin forms differing in molecular mass by approx 3k (Tierney et al., 1990). As a consequence, a range of inhibin molecular weight forms have been predicted and identified. The more generally known forms are 29k and 32k pro-αC (α*, α**), 32 and 36k inhibin (α*β, α**β), 45k (αN-αC), 50-60k (αN-αC/β and αN/proβ-β) and more than 90k (αN-αC/proβ-β).

The four major regions of *in vitro* bioactivity identified in hFF (33k, 36, 55k and 66k) in this study are attributed to mono and diglycosylated 31k inhibin A, as found in recombinant human inhibin A, αN-αC/β (55k) and either pro-αN-αC/β or αC/proβ-β (66k). These peaks are similar to those obtained by Robertson and colleagues (1997a) suggesting that the modified fractionation procedure presented here is a valid way to study the forms expressed in various pregnancy compartments.

In summary, this chapter details the immunoaffinity fractionation procedure, with modifications, used in the study of inhibin forms in pregnancy. Using the modifications to the immunoaffinity Prep-PAGE/electroelution procedure increases the efficacy of the Prep-PAGE procedure. The modifications to the fractionation procedure detailed above also increases practicability and enables the identification of many of the forms of inhibin in human fluids. These series of validation studies reported in this chapter set the foundations on which the studies detailed in the following chapters are based.

# CHAPTER FOUR

## Changes in Molecular Weight Forms of Inhibin A and Pro-αC in Maternal Serum During Human Pregnancy

## 4.1 SUMMARY

Maternal serum pools obtained from healthy women throughout normal pregnancy were fractionated by a combined immunoaffinity chromatography, preparative PAGE and electroelution procedure as detailed in Chapter Three. Inhibin A and the pro-αC region of the inhibin α subunit were determined in the eluted fractions by specific ELISAs and the profiles of immunoactivity characterised in terms of mol wt and % recovery. The mol wt patterns of inhibin A and pro-αC in serum during early pregnancy (<19 weeks gestation) showed peaks between 25-40k and ~60k, consistent with the presence of known mature and larger precursor inhibin forms. However, later in pregnancy (>19 weeks gestation), an increase in the proportion of smaller mol wt forms (from 2% to ~25%) of inhibin A and pro-αC of unknown structure were observed in the <30k and <25k regions, respectively.

To assess whether this change in mol wt distribution in late pregnancy was related to the method of serum collection, serum and plasma from women during early and late pregnancy were collected and snap frozen. Three pools of serum and plasma (one from early pregnancy, [12-15 weeks] and two from late pregnancy, [28-39 weeks]) were then fractionated as above. No differences in mol wt pattern of inhibin A and pro-αC were observed between serum and plasma pools obtained in early pregnancy. However, in late pregnancy, there was a reduction in the proportion of low mol wt forms between serum (25% inhibin A, 35% pro-αC) and plasma (11.9%, 17.4% respectively) but not to the low levels seen in early pregnancy. Incubation of iodinated 30k human inhibin with serum or plasma obtained from early or late pregnancy showed no evidence of cleavage, suggesting that 30k inhibin is not the cleavage precursor. It is postulated that the formation of small mol wt forms of both inhibin A and pro-αC is attributed to proteolytic changes, in part induced in the circulation during late gestation, and in part by the placenta prior to secretion. It is concluded that inhibin A and pro-αC are processed in late pregnancy by more than one mechanism to form low mol wt circulating forms of unknown structure.

## 4.2 INTRODUCTION

As detailed in Chapter One (section 1.2), inhibin is synthesized as two precursor chains linked by disulphide bonds, undergoing subsequent processing, through several recognized steps, to form the mature 30k form. Studies to date have highlighted two main proteolytic cleavage sites in the α subunit dividing the α subunit into three regions: Pro (amino acids (aa) 19-61), αN (aa 62-232) and αC (aa 233-366) (Mason et al., 1996a). One cleavage site has been identified within the $\beta_A$ subunit, giving two regions pro-β (aa 21-310) and $\beta_A$ (11 311-426) (Mason et al., 1996a). The subunits and their regions are diagrammatically represented in Chapter One (Figure 1.1, section 1.2). Studies exploring the forms of inhibin in biological fluids have identified inhibins ranging in size between the precursor and mature 30k (αC/β) forms (Robertson et al., 1997b), largely consistent with combinations of the above α and β subunit subregions. In addition, a processed form of the free α subunit, pro-αC, has been identified in high concentrations in biological fluids (Schneyer et al., 1990). The precursor forms of inhibin are intrinsically less bioactive compared to 30k inhibin or inactive requiring cleavage of the precursor sequences for bioactivity to be evident (Mason et al., 1996b). Robertson and colleagues (1995, 1996, 1997a,b) have shown the presence of these various forms of inhibin in human serum, plasma and follicular fluid. Little however, is known about the inhibin form(s) present in human maternal serum.

It is well established that there is a biphasic pattern to the levels of inhibin A and the α subunit (pro-αC), with an initial peak early in pregnancy followed by a rise in late pregnancy in maternal serum (Muttukrishna et al., 1995; Illingworth et al., 1996a; Fowler et al., 1998). In contrast, inhibin B levels are undetectable in early pregnancy (Wallace et al., 1997a), with only very low levels during the third trimester, (21pg/mL), (Fowler et al., 1998), probably reflecting the known 10% cross-reactivity of inhibin A in the assay (Wallace et al., 2000). It is believed that inhibin A throughout early and late pregnancy is of primarily feto-placental origin (Wallace et al., 1997a; Muttukrishna et al., 1997a) while pro-αC is a product of the corpus luteum during early pregnancy and the fetal placental unit during late pregnancy (Illingworth et al., 1996a; Rombauts et al., 1996; Muttukrishna et al., 1997a; Lockwood et al, 1997).

The aim of this study was to examine the forms of inhibin A and pro-αC in maternal serum and plasma across normal pregnancy using the improved fractionation procedure detailed in Chapter Three, in combination with specific ELISAs, in order to provide baseline information for comparisons with inhibin forms found associated with abnormal pregnancies (investigated in Chapters Six and Seven).

## 4.3 MATERIALS AND METHODS

### 4.3.1 Preparations

Maternal serum samples were collected at various stages of pregnancy from women attending the Antenatal Clinic, Monash Medical Centre (1998-1999). The women were all healthy with normal singleton pregnancies. Bloods were collected in plain serum-gel tubes (Greiner Labortechnik, Kremsmunster, Austria), stored at 4°C and centrifuged within 48h of collection. The serum was then stored frozen at –20°C. The sera were thawed and 7 serum pools, covering various gestation periods were prepared. Each pool consisted of equal aliquots from >20 women.

Serum (designated fresh serum) and plasma were also collected under more defined conditions from early pregnancy (eight women, 12-15 weeks) and late pregnancy (28-39 weeks, n=15 and 31-36 weeks, n=20). Blood was collected in serum-gel tubes (Greiner) on ice, allowed to clot at 4°C and centrifuged within 2h. Serum was stored frozen at –80°C. Plasma was collected on ice in EDTA-coated tubes (Greiner), centrifuged within 2h at 4°C and the plasma stored at –80°C.

This study was approved by the research and ethics committee of Monash Medical Centre (Clayton, Victoria, Australia). All women gave informed consent.

### 4.3.2 Fractionation Procedure

Serum and plasma were fractionated as detailed in Chapter Three. The percentage of recovered inhibin after each step (IA and Prep-PAGE) was calculated by assaying inhibin A and pro-αC before fractionation and then comparing levels after the IA and Prep-PAGE procedures. These recoveries are presented in Table 4.1 in the Results section (4.4.1).

### 4.3.3 Assays

#### *4.3.3.1 Inhibin A (αβA) ELISA*

The method of Groome and O'Brien (1993) was employed, with modifications (Groome et al., 1994), and with the human recombinant inhibin A (WHO 91/624) as standard. Details of the assay are presented in Chapter Three (section 3.2.4). The sensitivity of the assay was <15pg/mL.

#### *4.3.3.2 Pro-αC subunit ELISA*

The ELISA, as previously detailed by Groome and colleagues (1995), was employed. Details of the assay are presented in Chapter Three (section 3.2.4). The sensitivity of the assay was <6pg/mL.

#### *4.3.3.3 αC Immunofluorometric assay (αC IFMA)*

The αC IFMA, which detects all α-subunit containing forms, was performed by Tanneale Stephenson at the Prince Henry's Institute of Medical Research (PHIMR) and details of this assay has been published previously (Robertson et al., 1997a). In this study, it was noted that the electroelution buffer interfered in the αC IFMA with a marked loss of assay sensitivity. As the detection limit of this assay is poor, (>1000 pg/mL), only late pregnancy samples were tested. Each late pregnancy serum fraction was dialysed against water (3 days at 4°C) followed by a RP-HPLC step to remove the SDS. The sample was then lyophilized, reconstituted in assay buffer and assayed using 30k inhibin A (WHO 91/624) as standard.

### 4.3.4 Serum/plasma incubation with iodinated 30k inhibin A

Serum (4µl) or plasma (4µl) was incubated with 0.1M phosphate buffer, 0.1% bSA, 0.1% $NaN_3$, pH 7.2, containing 30,000cpm of iodinated 30k inhibin for 24h, 48h or 72h at room temperature. These serum/plasma samples had been collected and stored under the same conditions described above (section 4.3.1). An aliquot was then fractionated on SDS-PAGE in Tris/Tricine buffers using a Mini Protean Apparatus (BioRad). The gel was dried and the molecular wt pattern of radioactivity was visualized using a phosphoautoradiograph and phosphoimaging system (Molecular Dynamics, Sunnyvale, CA).

### 4.3.5 Statistical/Data Analysis

The mol wts of the eluted Prep-PAGE fractions were calculated as described previously (Chapter Three, section 3.2.5). Regression analysis (fraction number vs log mol wt) of the 3 protein standard markers used (soybean trypsin inhibitor 23.5k, bovine carbonic anhydrase 53k and bSA 82k) gave correlation coefficients >0.995. The resulting line of best fit was used to determine the mol wts of the eluted fractions.

The mol wt values of peak tubes of immunoactivity from multiple runs were combined (mean ± SD) and from their distribution, activity regions were defined (see Table 4.2). The immunoactivity levels/fraction are also presented as a percentage of recovered activity in Figures 4.1-4.5. Changes in the proportions of inhibin A and pro-αC in the <30k and >40k and <25k and >40k mol wt regions respectively, were analysed using Spearman's Rank correlation (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, USA).

## 4.4 RESULTS

### 4.4.1 Recoveries

Recoveries of inhibin A and pro-αC immunoactivities for the series of sera and plasma are presented in Table 4.1.

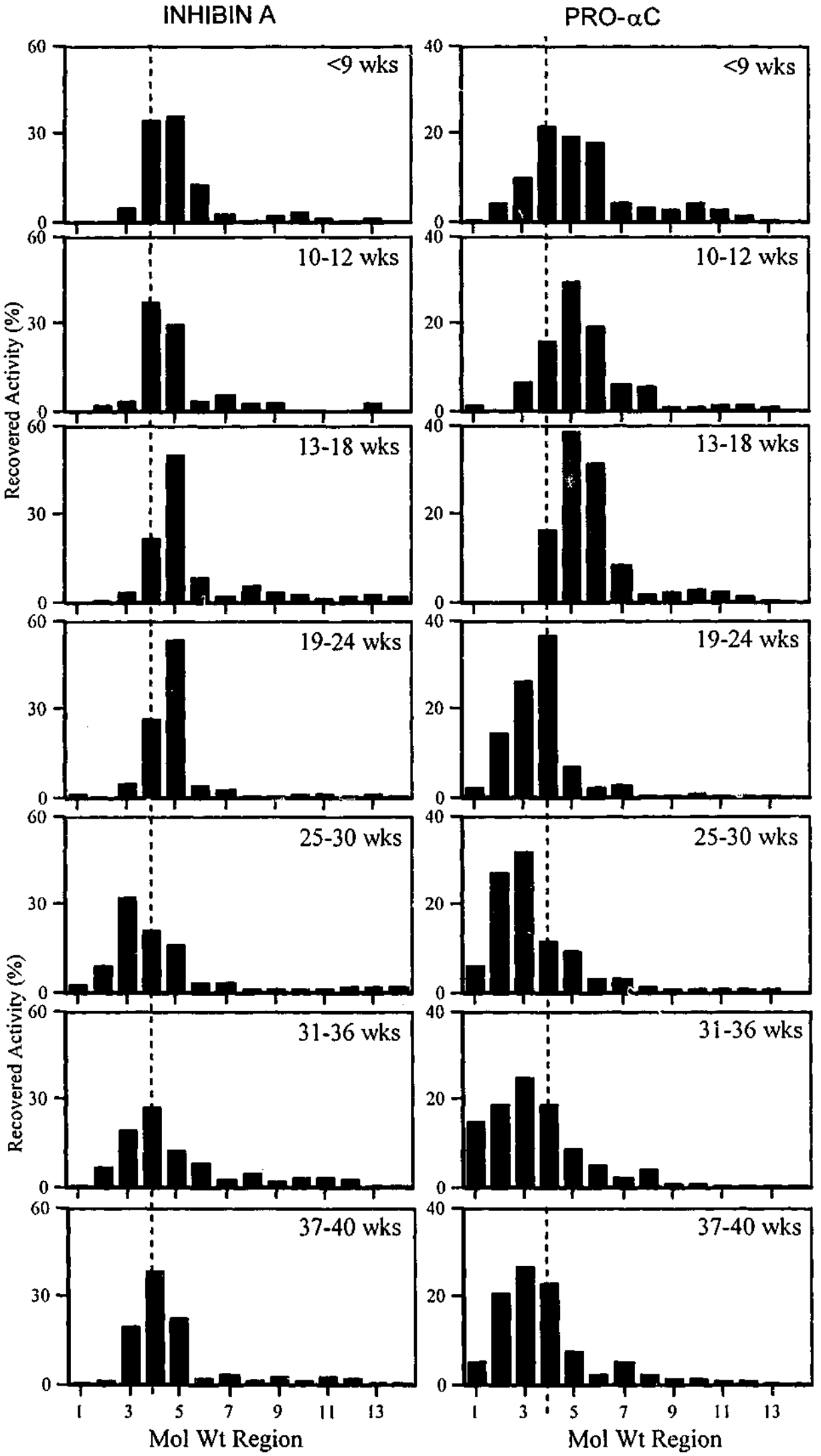
**Table 4.1:** Recoveries (%) of inhibin A and pro-αC through the immunoaffinity (IA) and preparative PAGE-fractionation procedure of serum/plasma. Mean ± SD, n= number of fractionations

| | Sample | n | IA | Prep-PAGE | Combined IA + Prep-PAGE |
|---|---|---|---|---|---|
| **Inhibin A** | Serum | 10 | 60.7 ± 15.6 | 58.3 ± 20.2 | 32.3 ± 18.7 |
| | Plasma | 3 | 57.2 ± 7.8 | 53.4 ± 10.2 | 27.7 ± 8.2 |
| **Pro-αC** | Serum | 10 | 50.7 ± 18.2 | 45.3 ± 22.7 | 25.3 ± 18.5 |
| | Plasma | 3 | 60.4 ± 4.9 | 47.8 ± 10.8 | 28.3 ± 7.5 |

### 4.4.2 Pregnancy Serum

The patterns of inhibin A and pro-αC immunoactivity were determined in 7 individual serum pools collected at specific stages throughout normal pregnancy (Figure 4.1).

**Figure 4.1:** Molecular weight distribution of inhibin A and pro-αC immunoactivities of single serum pools collected throughout pregnancy and processed through the combined IA/prep-PAGE procedure. The vertical dashed line refers to the mol wt of 31.4k inhibin A used as a reference (see Chapter Three, section 3.3.1.1). Mol wt values corresponding to the mol wt regions are presented in Table 4.2.


INHIBIN A
PRO-αC
<9 wks
10-12 wks
13-18 wks
19-24 wks
25-30 wks
31-36 wks
37-40 wks
Recovered Activity (%)
Mol Wt Region
1
3
5
7
9
11
13
0
30
60
20
40

In the main, similar mol wt forms of inhibin A and pro-αC were identified throughout pregnancy (table 4.2) although inhibin A forms with mol wts <30k and pro-αC forms <25k (ie. mol wts less than the recognized mol wts for ~30k recombinant human inhibin and pro-αC) were identified in late pregnancy (>19 weeks) (Table 4.2; Figure 4.1).

**Table 4.2:** Apparent molecular wt (k) of peak tubes of inhibin A and Pro-αC immunoactivity, determined following fractionation of serum obtained from different stages of pregnancy. Mean±SD (n=number of fractionations in which a particular mol wt form was clearly present)

| | Mol wt | Early Pregnancy (<19 weeks) | | Late Pregnancy (>19 weeks) | |
|---|---|---|---|---|---|
| **Region** | **Range (k)** | **Inhibin A** | **Pro-αC** | **Inhibin A** | **Pro-αC** |
| 1 | 15.1-20.0 | | | | 20.1 ± 0.7 (n=4) |
| 2 | 20.1-25.0 | | | 21.7 ± 1.1 (n=3) | 22.3 (n=2) |
| 3 | 25.1-30.0 | | 27.4 ± 0.8 (n=5) | 27.2 ± 1.3 (n=4) | 27.3 ± 0.9 (n=5) |
| 4 | 30.1-35.0 | 31.2 ± 0.5 (n=3) | 32 (n=2) | 31.9 ± 1.3 (n=5) | 31.7 ± 0.8 (n=3) |
| 5 | 35.1-40.0 | 37.7 ± 1.1 (n=5) | 38.3 ± 0.4 (n=5) | 36.7 ± 1.3 (n=6) | 36.2 ± 1.2 (n=3) |
| 6 | 40.1-45.1 | | 42.2 (n=2) | | |
| 7 | 45.1-50.0 | | | | 48.1 ± 1.6 (n=5) |
| 8 | 50.1-55.0 | | | | |
| 9 | 55.1-60.0 | | | | 57.9 (n=2) |
| 10 | 60.1-65.0 | 63.5 ± 2.5 (n=3) | 64.1 ± 2.4 (n=4) | 63.7 ± 2.4 (n=3) | |
| 11 | 65.1-70.0 | | | | 65.9 (n=2) |
| 12 | 70.1-75.0 | | | | |
| 13 | 75.1-80.0 | | | 75.9 ± 0.3 (n=3) | |
| 14 | 80.1-85.0 | 81.8 (n=2) | | | |

In order to explore this apparent change in mol wt distribution across pregnancy, the mol wt profile for inhibin A and pro-αC was divided into three mol wt regions. Inhibin A: <30k, 30-40k and >40k and pro-αC: <25k, 25-40k and >40k (figures 4.2 and 4.3). A marked change in the proportion of forms <30k inhibin A and <25k pro-αC in midpregnancy was observed, with the proportion of immunoactivity recovered increasing from <5% prior to 19 weeks to ~25% after 19 weeks (figures 4.2 and 4.3).

To investigate if the larger mol wt forms of inhibin (>40k) are the precursors to the <30k and <25k inhibin A and pro-αC forms, a Spearman's Rank correlation was undertaken, comparing the <30k and >40k inhibin A and <25k and >40k pro-αC forms, respectively. A significant correlation ($r= -0.89$, $p=0.012$) was observed for inhibin A but not pro-αC ($r=0.04$, $p=0.93$).

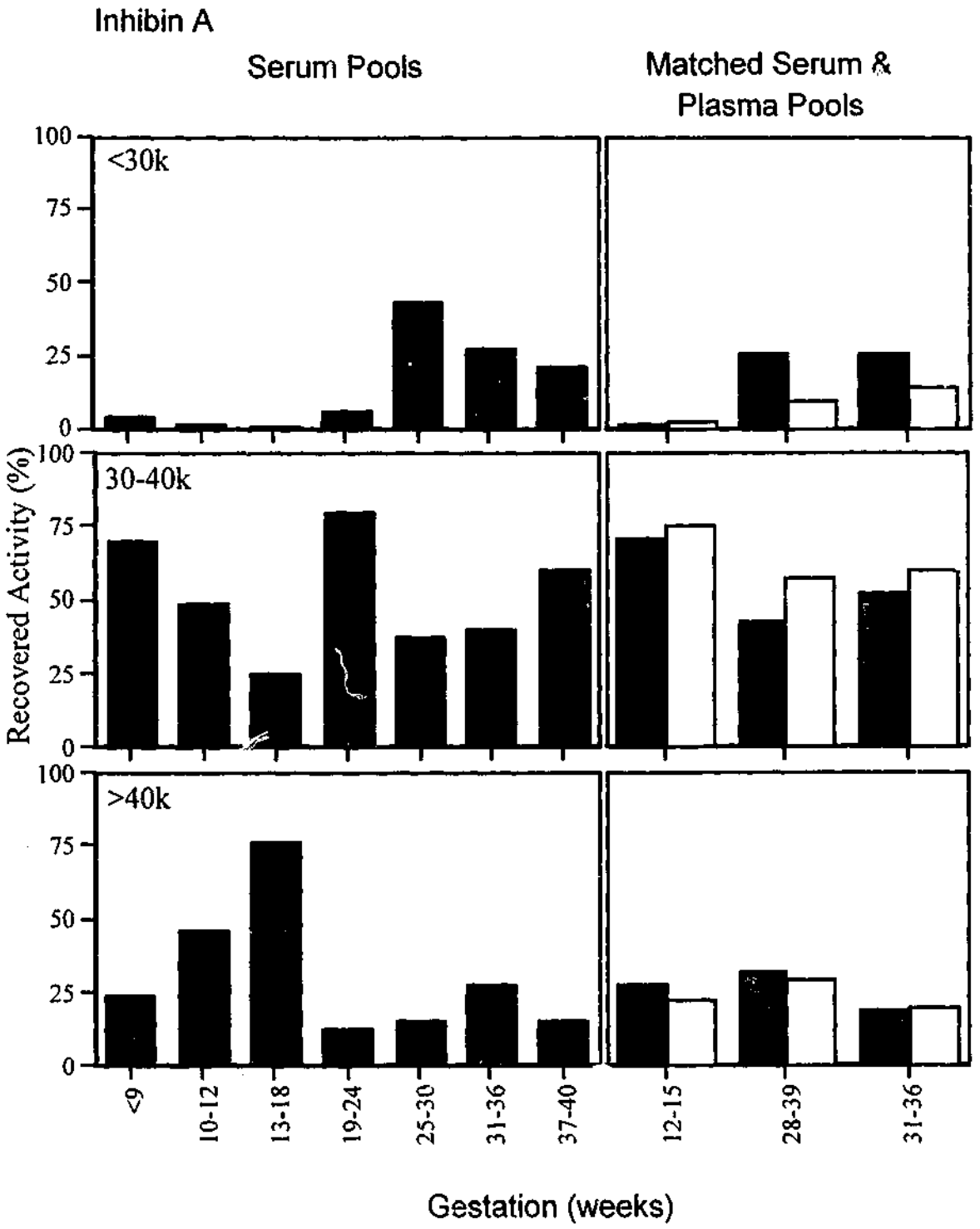



**Figure 4.2:** Changes in the proportion of recovered inhibin A in different mol wt regions throughout pregnancy. The data represent results from 7 serum pools (■) collected routinely in an antenatal clinic and three pools of matched serum (grey bars) and plasma (□) collected under defined conditions. The pools were fractionated through the combined IA/prep-PAGE procedure.

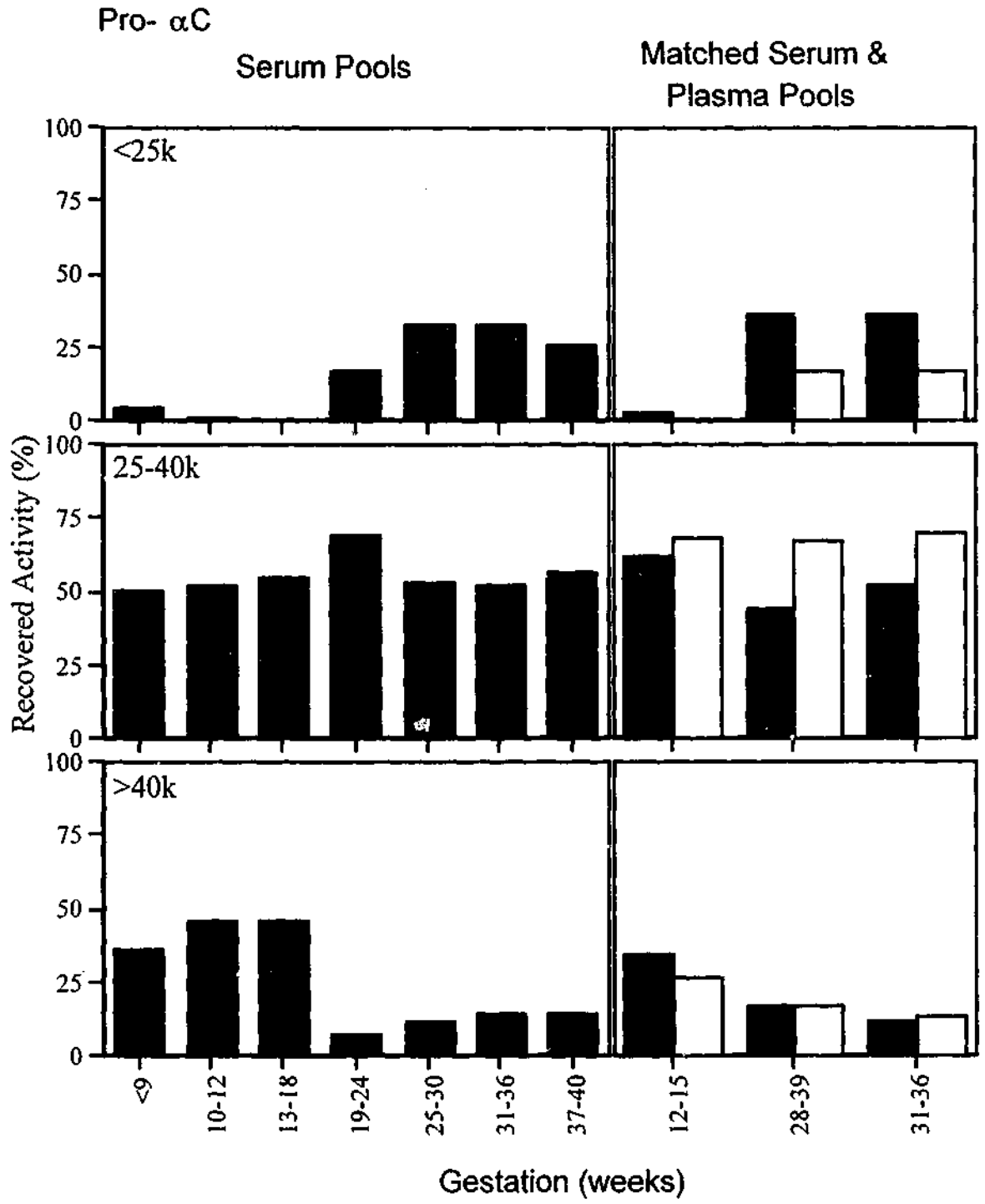
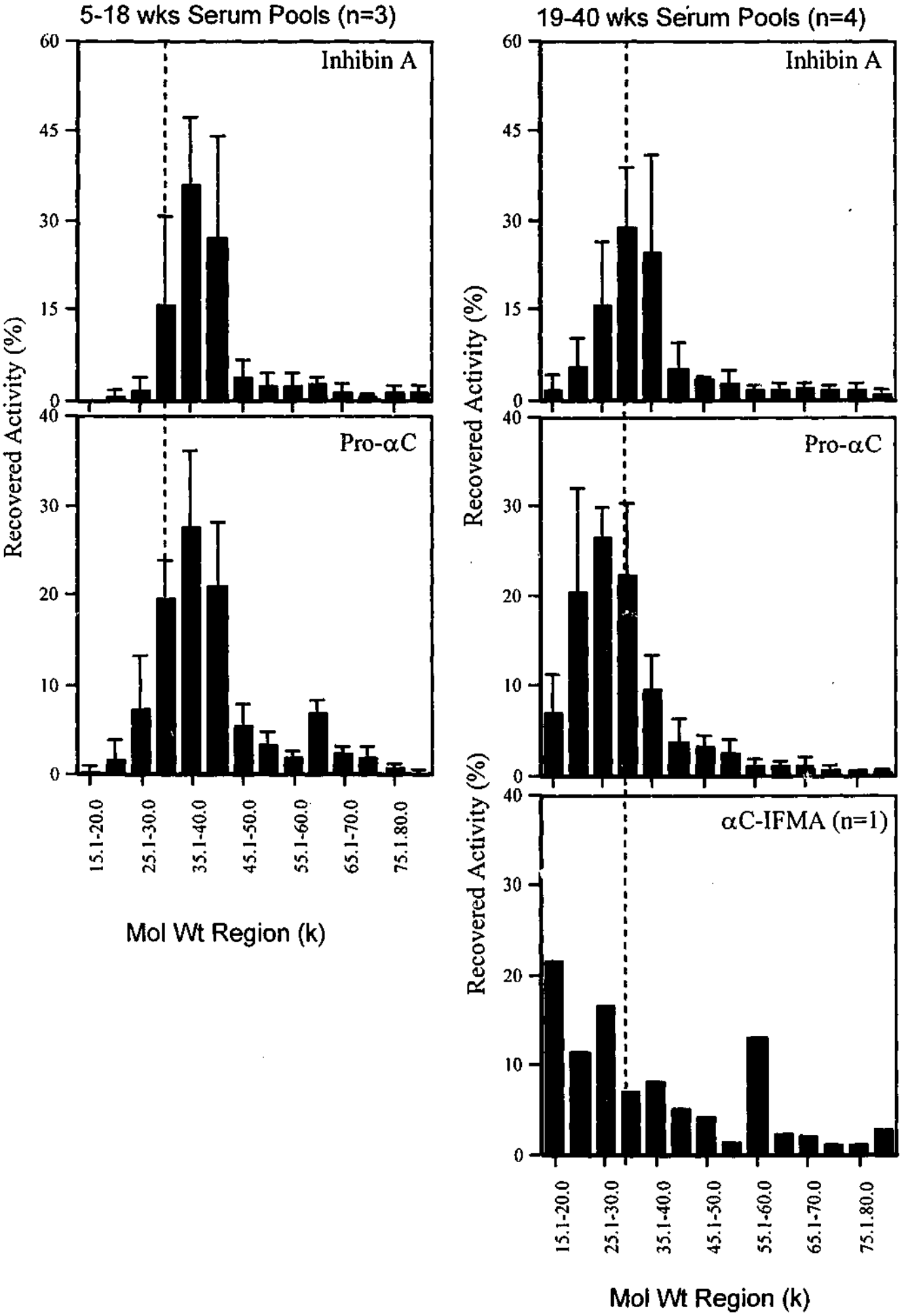



**Figure 4.3:** Changes in the proportion of recovered pro-αC in different mol wt regions throughout pregnancy. The data represent results from 7 serum pools (■) collected routinely in an antenatal clinic and three pools of matched serum (grey bars) and plasma (□) collected under defined conditions. The pools were fractionated through the combined IA/prep-PAGE procedure.

Based on this apparent shift in size at 19 weeks, the mol wt patterns for serum pools obtained before and after 19 weeks were separately combined and the pooled data are

**Figure 4.4:** Mean ± SD molecular weight distribution of immunoactivity determined by inhibin A, Pro-αC and αC-IFMA in early- (5-18 weeks) and late- (19-40 weeks) pregnancy serum processed through the combined IA/prep-PAGE procedure. αC-IFMA assay was not performed on early pregnancy samples because of poor assay sensitivity (see section 4.3.3.3). The vertical dashed line refers to the mol wt of 31.4k recombinant inhibin A used as reference.

presented in Figure 4.4. The profile of α subunit-containing forms in one pool of late pregnancy serum as determined by the αC-IFMA also showed elevated levels of immunoactivity in the low mol wt regions (figure 4.4 below).



### 4.4.3 Pregnancy serum vs plasma

One explanation for the apparent reduction in mol wts of inhibin A and pro-αC during late pregnancy is the effects of proteolysis induced during serum collection and storage. The pregnancy serum pools studied were collected as part of a clinical service, and hence stored at 4°C for up to 48h before storage of serum at –20°C. Under these storage conditions, cleavage may occur. To explore this possibility further, fresh serum and plasma obtained under rapid and chilled collection conditions were obtained from women during early (13-18 weeks) and late pregnancy (two separate pools, 28-39 and 31-36 weeks) and fractionated as above. As seen in Figures 4.3-4.5, inhibin A and pro-αC profiles in early pregnancy serum and plasma showed little change in mol wt pattern. However, in late pregnancy, plasma showed lower levels of <30k inhibin A and pro-αC compared to the original serum profiles but not as low as that seen during early pregnancy (Figures 4.3-4.5). The proportion of >40k forms was less affected between serum and plasma.

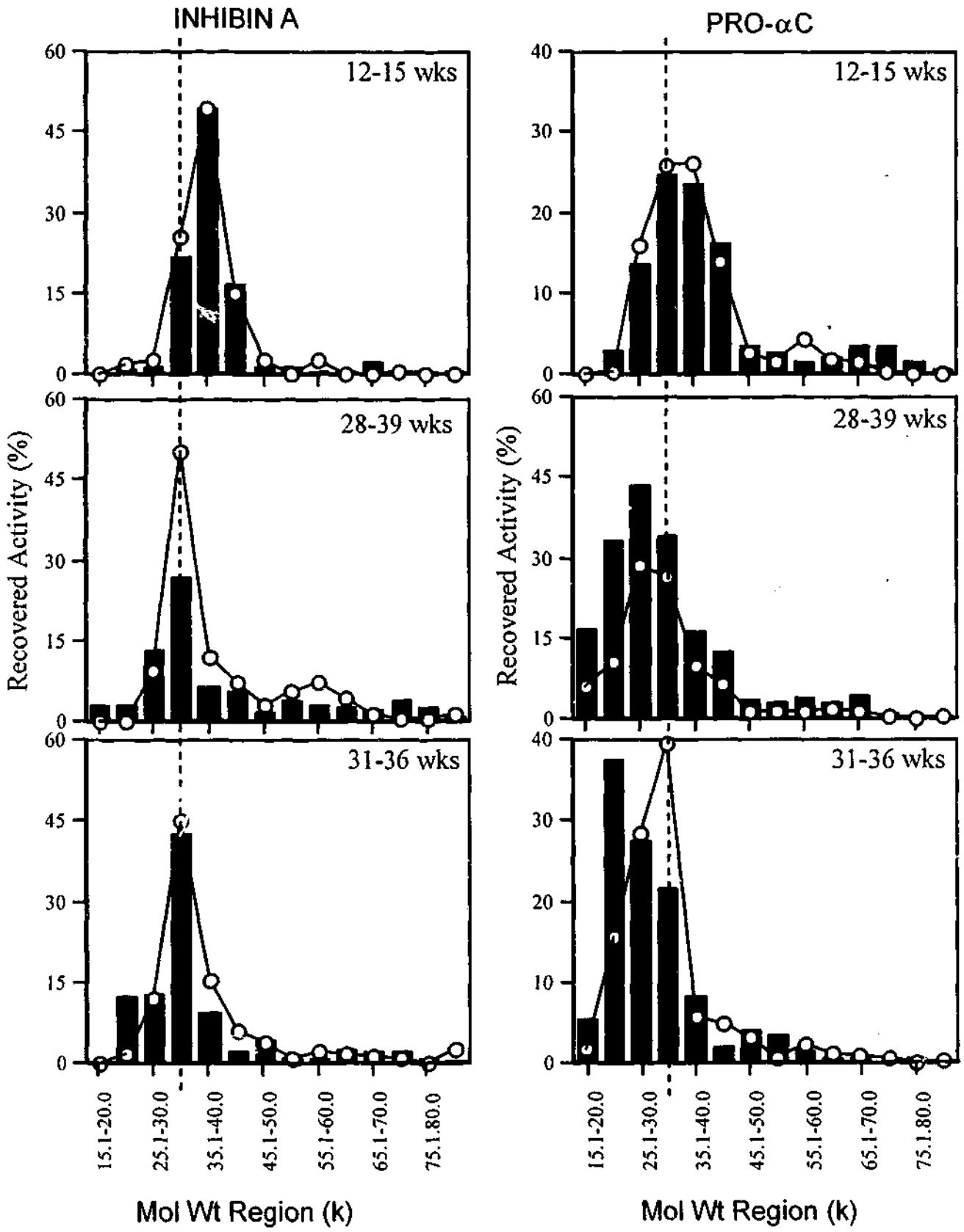



**Figure 4.5:** Molecular weight distribution of inhibin A and pro-αC in matched serum (■) and plasma (○) samples, collected under defined conditions (see section 4.3). The pools were fractionated through the combined IA/prep-PAGE procedure. The vertical dashed line refers to the mol wt of 31k recombinant inhibin A used as reference.

To explore whether pregnancy serum contains proteases which cleave inhibin, iodinated 30k inhibin was incubated with serum or plasma obtained from early and late pregnancy under various conditions. No changes in mol wt of iodinated inhibin were seen under any of the incubation conditions used (data not shown).

## 4.5 DISCUSSION

The objectives of this study were to describe inhibin A and pro-αC forms present in serum during pregnancy and to establish if the mol wt profiles of inhibin A and pro-αC in serum were modified during human pregnancy. The results show peak inhibin A forms of 20-27k, 31-37k, 63-65k and 75-81k, and peak pro-αC forms of 20-27k, 31-36k, 42-48k and 57k-65k throughout pregnancy, consistent with reports by Khalil et al (1995), demonstrating high and low inhibin molecular weight forms in pregnancy serum, but contrary to the findings of Muttukrishna et al (1995) who reported mainly 31k inhibin A in pregnancy serum. This may be due to the method used by Muttukrishna and colleagues (1995), gel filtration chromatography, which has limited resolution and may not readily detect relatively low levels of the small, or large, inhibin A and pro-αC forms.

The finding that there is a major increase in the proportion of small mol wt forms of inhibin A and pro-αC in maternal serum during late pregnancy compared to early pregnancy and non-pregnancy (luteal phase serum data presented in Chapter Three) is novel. No studies to date have examined the changes in inhibin molecular weight forms in association with increasing gestation. The observations that there was less of the small mol wt forms in plasma, collected under more stringent conditions, and that sera recoveries were not significantly different from plasma, suggests that, in late pregnancy, the changes in mol wt forms are due to proteolysis in serum and not differential losses in plasma and serum.

The structures of the small mol wt forms of inhibin A and pro-αC present in late pregnancy are unclear. The similar change in mol wt pattern during pregnancy for inhibin A and pro-αC suggests that there is a common mechanism probably related to cleavage of the α subunit. Mature 30k inhibin consists of two known forms (32 and 34k), the differences attributed to mono- and di- glycosylated forms of the α subunit

($\alpha^{**}\beta_A$ and $\alpha^{*}\beta_A$) (Mason et al., 1996a; Tierney et al., 1990). In the present study, these forms (31-32k and 37k) are present throughout pregnancy. Similarly, the observed 32 and 27k forms of pro-αC most likely refer to mono- and di-glycosylated pro-αC forms. What are the likely structures of smaller forms?

One possibility is that the 27.2k inhibin A form found in late pregnancy serum is deglycosylated inhibin A. However there is no ready explanation for the much smaller 21.7k inhibin A form. It should be noted that these forms were determined by an inhibin A ELISA using as one of the two antibodies, an α subunit directed monoclonal antibody (R1) where the epitope is located within the N terminal region of the αC subunit (Groome et al., 1994), and the other, a $\beta_A$ subunit directed antibody. Thus, the 21.7k form must be dimeric inhibin A. Although <30k inhibin forms have been isolated from human and testicular sources in which the terminal 17 aa of the N terminus of the αC region has been deleted (Hasegawa et al., 1994; Bardin et al., 1987), the reduction in length is insufficient to account for the reduced size. Furthermore, if a larger portion of the αC region was excised (say, terminal 32 aa), the epitope region for the α subunit Mab also would be excised, rendering it undetectable in the assay used. A similar situation applies with pro-αC where mol wt forms of 22.3k and 20.1k were identified. One would need to postulate that to account for the marked reduction in size of both inhibin A and pro-αC, that either an internal sequence of the αC region has been deleted or that a large portion of the N terminal region of the α-subunit has been cleaved and that the α subunit epitope is still present at least in part. Cleavage of the pro-sequence, the carboxy terminus (although disulphide linked), and $\beta_A$ subunit cannot be excluded. The possibility of reduced glycosylation by the placenta leading to deglycosylated, and therefore reduced, sizes of inhibin α subunit is also a possibility. Further studies are required to define the structure of these inhibins.

The low mol wt forms were readily detected by the αC IFMA. This assay employed polyclonal antisera which was directed to multiple epitopes on the α-subunit (Robertson et al., 2001) and may detect forms not detected with the ELISAs. Small mol wt forms of inhibin have been detected previously in fractionated post-menopausal plasma (Robertson et al., 1997b). These forms were attributed in part to

the presence of the free α-subunit although it may also represent small mol wt forms as found in pregnancy plasma.

The increased small molecular weight forms in late pregnancy may be due to increased proteolysis with advancing gestation. A number of studies have shown the presence of increased proteolytic activity in pregnancy serum. A serum protease that cleaves IGF binding protein 4 and 5 is stimulated by pregnancy with highest levels in late gestation (Byun et al., 2000; Claussen et al, 1994). Similarly, an hCGβ subunit nicking enzyme (Kardana and Cole, 1994) responsible for the formation of degraded hCG has been specifically detected in pregnancy serum.

The normal cleavage of inhibin at dibasic peptide sites (Pro-αN, αN-αC, proβ-β regions) is believed to be caused by intracellular furin-like serine peptidases. Thus, the cleavage of precursor forms (>40k) observed in late pregnancy may be attributed to the effects of placental furins. Previous studies by McLachlan et al (1986b) showed that 58k bovine inhibin A ($\alpha N\text{-}\alpha C/\beta_A$) was cleaved in serum to form 30k inhibin by serine peptidase-like activity with pH and inhibitor specificity similar to furin (Robertson et al., 1987b) but that 30k inhibin itself was not further metabolized by serum. The observation that 30k inhibin was not cleaved in that study is supported by the observations in the present study. It is also interesting to note that a significant correlation was observed between the <30k and >40k regions for inhibin A but not pro-αC, suggesting that the larger mol wt forms of inhibin, rather than the 30k inhibin forms, are the precursors to the <25k inhibin forms. Unfortunately, no stocks of purified forms of high mol wt inhibin were available to explore whether the >40k form is the precursor of the small mol wt forms. Cleavage of high mol wt forms of inhibin may occur in either the placenta or circulation.

The possibility that artefacts in the fractionation procedure may be responsible for the formation of the small mol wt forms is unlikely, as discussed in Chapter Three. The overall recovery of inhibin activity throughout the various purification steps ranged from 25-32% with recoveries of 50-60% in the immunoaffinity step and 45-58% in the Prep-PAGE/electroelution step. These reduced recoveries are not attributed to differential losses of particular inhibin forms at particular steps as inhibin immunoactivity is not detectable in immunodepleted serum (Robertson et al., 1985),

indicating that >95% of the inhibin binds to the immunosupport. The losses at the Prep-PAGE step have been previously shown by Robertson et al (1985) to be similar for both high and low mol weight forms. The observed losses in this study are therefore attributed to non-specific/irreversible binding to the various supports and to the difficulty in obtaining quantitative recoveries in the electroelution step due to limitations in the methodology.

Future studies will need to address the biological significance of these small mol wt forms during pregnancy. At this stage, it is unclear if the increase in small mol wt forms are attributed to the normal progression of pregnancy or some other undefined clinical difference in the patient groups that is associated with altered inhibin processing. It is also unclear if the small mol wt forms of inhibin A are bioactive. Establishing the immunoactivity of the small mol wt forms was not feasible as a mass of 2-3μg of purified protein would have been required, needing litres of serum to obtain enough of the small mol wt forms. While necessary for appreciating the significance of the changes described, quantitative studies and the potential physiological role/significance of individual isoforms must await purification of adequate amounts of material allowing identification of structures and assessments of bio- and immunoactivity.

# CHAPTER FIVE

## Placental Inhibin A and Pro-αC Molecular Weight Forms

## 5.1 SUMMARY

To investigate if the gestation-related changes in circulating maternal serum inhibin molecular weight forms (Chapter Four) arose from altered placental production, pooled placental extracts, derived from normal healthy singleton pregnancies in the 1st, 2nd and 3rd trimesters of pregnancy, were purified and fractionated by a combined immunoaffinity chromatography, preparative PAGE and electroelution procedure as previously described (Chapter Three). Inhibin A, inhibin B and the pro-αC region of the inhibin α subunit were measured in the eluted fractions by specific ELISAs, with the profiles of immunoactivity characterised in terms of molecular weight and percentage recovery. Inhibin B was undetectable in all samples. Molecular weight peaks of 36k, 75k and 97k for inhibin A and 29k, 55k and 97k for pro-αC were detected in placental extracts across all trimesters. Compared to first trimester tissue, placental extracts from the second and third trimesters of pregnancy showed a limited increase in the proportion of smaller molecular weight forms of inhibin A (from 0.1% in the first trimester to 7.3% in term tissue) and pro-αC (1.1% to 5.5%, respectively), but these levels did not mirror the significantly higher proportions of small molecular weight forms observed in maternal serum in late pregnancy (Chapter Four).

These data suggest that the presence of small molecular weight forms of both inhibin A and pro-αC in maternal serum is only partially attributed to placental production and/or secretion. It is concluded that inhibin A and pro-αC inhibins in maternal serum are processed in late pregnancy by more than one mechanism to form low molecular weight circulating forms.

## 5.2 INTRODUCTION

As detailed in Chapter Four, the relative amounts of the various mol wt forms of inhibin A and pro-αC change in late pregnancy such that there is a 20% increase in the proportion of smaller mol wt forms (<30k and <25k, respectively) after 19 weeks' gestation. Whether this change is due to altered placental secretion or to post-secretory cleavage, as has been demonstrated in non-pregnancy serum (McLachlan et al., 1986b), remains unknown and was the underlying investigation of this study. Accordingly, the forms of inhibin A, inhibin B and pro-αC were examined in 1$^{st}$, 2$^{nd}$ and term placental extracts using the fractionation procedures previously detailed and validated (Chapter Three) to define the contribution of the placenta to the changing molecular weight forms of inhibin observed in maternal serum.

## 5.3 MATERIALS & METHODS

### 5.3.1 Sample collection

Placentae from 17 first (6-11 weeks) and 11 second trimester (15-18 weeks) singleton pregnancies were collected from normal healthy women with an apparently normal pregnancy undergoing surgical termination of pregnancy for psychosocial reasons. Cervical pre-treatment with 400μg vaginal misoprostil (Cytotec, Searle, Crows Nest, NSW) was administered 2-3 hours before surgery which was performed under general anaesthesia. The first trimester terminations of pregnancy were performed by vacuum aspiration under general anaesthesia. Second trimester terminations of pregnancy were performed by dilation of the cervix and surgical evacuation (D&E), also performed under general anaesthesia. Term (37-40 weeks) placentae were obtained immediately after an uncomplicated spontaneous vaginal delivery of a singleton infant from three normal healthy women. All women gave written, informed consent. The studies had the approval of the Monash Medical Centre Human Research and Ethics Committee.

### 5.3.2 Tissue preparation

Blood was washed off all placental tissues thrice with ice-cold 0.1M PBS and the placental villi were dissected free of fetal membranes before being frozen in liquid nitrogen and stored at –80°C for homogenization. Placental tissue was homogenized using a modification of the procedure performed by de Kretser et al (1994). Each

sample was thawed and weighed. Placental tissue (35g) from each gestational group was then minced into small pieces before homogenization in an equal volume of 0.2M phosphate buffer (pH 7.4), containing protease inhibitors, 20mM EDTA and 2mM phenylmethylsuphonylfluoride (pMSF). The homogenate was centrifuged at 10,500 x *g* for 15 mins at 4°C, the resulting pellet discarded, and the supernatant centrifuged further at 10,500 x *g* for a further 10 mins at 4°C. The process was repeated once more and the final resulting supernatant was syringe-filtered through a 200μm nylon filter and stored at –20°C until fractionated.

### 5.3.3 Fractionation Procedure

The placental preparations above were fractionated (30mls) as previously described (Chapter Three, section 3.2.3), using the immunoaffinity (IA)/prep-PAGE procedure. The mol wts of the eluted Prep-PAGE fractions were calculated using regression analysis against three protein markers (soybean trypsin inhibitor 23.5k, bovine carbonic anhydrase 53k and bSA 82k) as described in detail in Chapter Three (section 3.2.5).

### 5.3.4 Inhibin Assays

The fractions were assayed for inhibin A, inhibin B and pro-αC as described previously (Chapter Three, section 3.2.4).

### 5.3.5 Statistical Analyses

Data is presented as mean ± SD unless otherwise stated. To facilitate comparisons between chromatograms, activity regions were defined for each placental profile as detailed in Chapter Three (section 3.2.5). Changes in the proportions of inhibin A and pro-αC in the <30k and >40k and <25k and >40k mol wt regions, respectively, were analysed using a *t*-test (Statview 4.1, Abacus Inc, CA).

## 5.4 RESULTS

### 5.4.1 Recoveries

Recoveries of inhibin A and pro-αC immunoactivity for the series of placental extracts are presented in Table 5.1.

**Table 5.1:** Recoveries (%) of inhibin A and pro-αC through the immunoaffinity (IA) and prep-PAGE fractionation of placental extracts.

| | N | IA | Prep-PAGE | Combined IA + Prep-PAGE |
|---|---|---|---|---|
| **Inhibin A** | 8 | 44.3 ± 20.2 | 30.1 ± 12.9 | 37.3 ± 17.6 |
| **Pro-αC** | 8 | 41.3 ± 18.2 | 29.3 ± 13.2 | 34.4 ± 16.5 |

These recoveries are similar to those published by Robertson et al (1995) and that achieved for serum/plasma (Chapter Four).

### 5.4.2 Inhibin profiles in placenta across pregnancy

Examples of the inhibin A and pro-αC profiles obtained in placental extracts from the 1st and 2nd trimesters and term pregnancy are presented in Figure 5.1 below. Both inhibin A and pro-αC levels were lowest in 2nd trimester tissues (Figure 5.1).

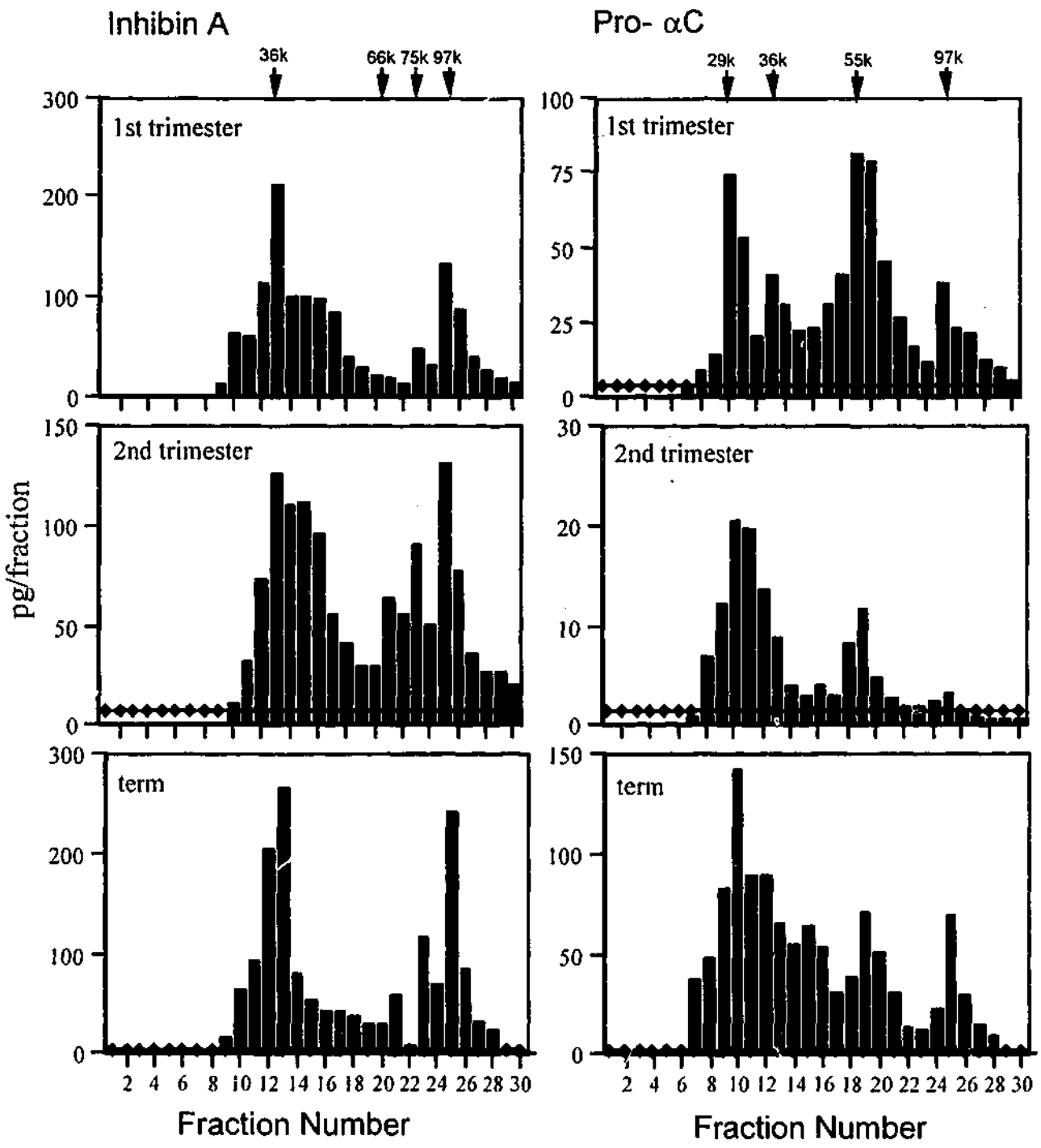



**Figure 5.1:** Molecular weight profiles of inhibin A and pro-αC immunoactivities in 1st and 2nd trimester and term placental pools fractionated through a combined IA/prep-PAGE procedure.

Similar mol wt forms of inhibin A were identified throughout pregnancy, with the ~36k form (α-$\beta_A$ dimer), the ~75k form (most likely αC/proβ-$\beta_A$) and the ~97k form (most likely αN-αC/Proβ-$\beta_A$) common in all three trimesters of pregnancy (Table 5.2). The 66k inhibin A form (Pro-αN-αC/$\beta_A$) was greatly reduced in 1st trimester tissue (Figure 5.1).

**Table 5.2:** Mean ± SD peak inhibin A molecular weights in 1st, 2nd and term placental extracts. n=number of fractionations.

| Mol Wt Range (k) | 1st trimester | 2nd trimester | Term |
|---|---|---|---|
| 35.1-40.0 | 36.3 (n=2) | 37.3 ± 1.4 (n=3) | 36.6 ± 2.1 (n=3) |
| 60.1-65.0 | | 66.4 ± 1.0 (n=3) | 66.8 ± 0.8 (n=3) |
| 75.1-80.0 | 76.1 (n=2) | 77.6 ± 2.1 (n=3) | 75.9 ± 0.3 (n=3) |
| >85.1 | 97.3 (n=2) | 98.5 ± 1.1 (n=3) | 97.9 ± 1.9 (n=3) |

In the pro-αC profiles, the monoglycosylated form of Pro-αC (~29k) and the ~55k Pro-αN-αC form were common structures in all three trimesters of pregnancy (Table 5.3), while the diglycosylated Pro-αC form (~36k) was greatly reduced in 2nd trimester and term tissue (Figure 5.1). Similarly, the ~97k form, present in both 1st trimester and term tissues, was greatly reduced in 2nd trimester tissues (Figure 5.1).

**Table 5.3:** Mean ± SD peak pro-αC molecular weights in 1st, 2nd and term placental extracts. n=number of fractionations.

| Mol Wt Range (k) | 1st trimester | 2nd trimester | Term |
|---|---|---|---|
| 25.1-30.0 | 29.2 (n=2) | 28.4 ± 1.3 (n=3) | 28.5 ± 0.9 (n=3) |
| 35.1-40.0 | 36.1 (n=2) | | |
| 50.1-55.0 | 54.6 (n=2) | 53.6 ± 1.3 (n=3) | 52.1 ± 2.1 (n=3) |
| >85.1 | 96.3 (n=2) | | 98.9 ± 0.4 (n=3) |

Additionally, inhibin A forms with mol wts <30k and pro-αC forms <25k, representing mol wts less than those recognised for recombinant inhibin and pro-αC (Chapter Three, section 3.3.1.1), were identified in the 2nd and 3rd trimester placental extracts. The distribution of these forms of inhibin A and pro-αC across the three

trimesters of pregnancy is presented in Table 5.4 below, where changes in three mol wt regions are highlighted for inhibin A (<30k, 30-40k and >40k) and pro-αC (<25k, 25-40, and >40k).

**Table 5.4:** Proportions (% mean ± SD) of inhibin A and pro-αC forms recovered in the various mol wt ranges (inhibin A <30k, 30-40k and >40k; Pro-αC <25k, 25-40k and >40k) in placentae. n= number of pools fractionated, with up to 9 samples per placental pool.

| | | **Inhibin A** | | | **Pro-αC** | | |
|---|---|---|---|---|---|---|---|
| **n** | **weeks** | **<30k** | **30-40k** | **>40k** | **<25k** | **25-40k** | **>40k** |
| 2 | 6-11 | 0.1 | 19.7 | 80.2 | 1.1 | 24.9 | 74 |
| 3 | 15-18 | 0.5±0.3 | 18.4±8.1 | 81.1±7.9 | 5.5±4 | 45.1±6 | 49.4±8.2 |
| 3 | 37-40 | 7.3±2.4 | 36.2±4.9 | 56.5±4.3 | 2.2±1.9 | 28.4±11.9 | 69.4±10.1 |

In order to compare these proportions with that present in maternal serum, the serum profiles from Chapter Four were pooled into 1$^{st}$ (n=2), 2$^{nd}$ (n=2) and 3$^{rd}$ trimesters (n=3) of pregnancy and the proportions of <30k, 30-40k, >40k inhibin A and <25k, 25-40k, >40k Pro-αC forms were similarly calculated. Figure 5.2 and Table 5.4 show that although the proportion of forms <30k inhibin A and <25k pro-αC increased in term placental tissue, with the proportion of immunoactivity recovered increasing from 0.1% prior to 18 wks to ~7.3% after 36 wks for inhibin A and from 1.1% to ~2.2% for pro-αC, the small mol wt forms of inhibin A and pro-αC in late pregnancy serum were significantly more abundant than in placenta (*t*-test, P=0.006). Conversely, the large mol wt forms (>40k) of inhibin A and pro-αC were more abundant in term placenta (inhibin A, 56.5±4.3; pro-αC, 69.4±10.1) than late maternal serum (inhibin A, 30.1±12.7; pro-αC, 15.1±1.9) (*t*-test, P=0.01).

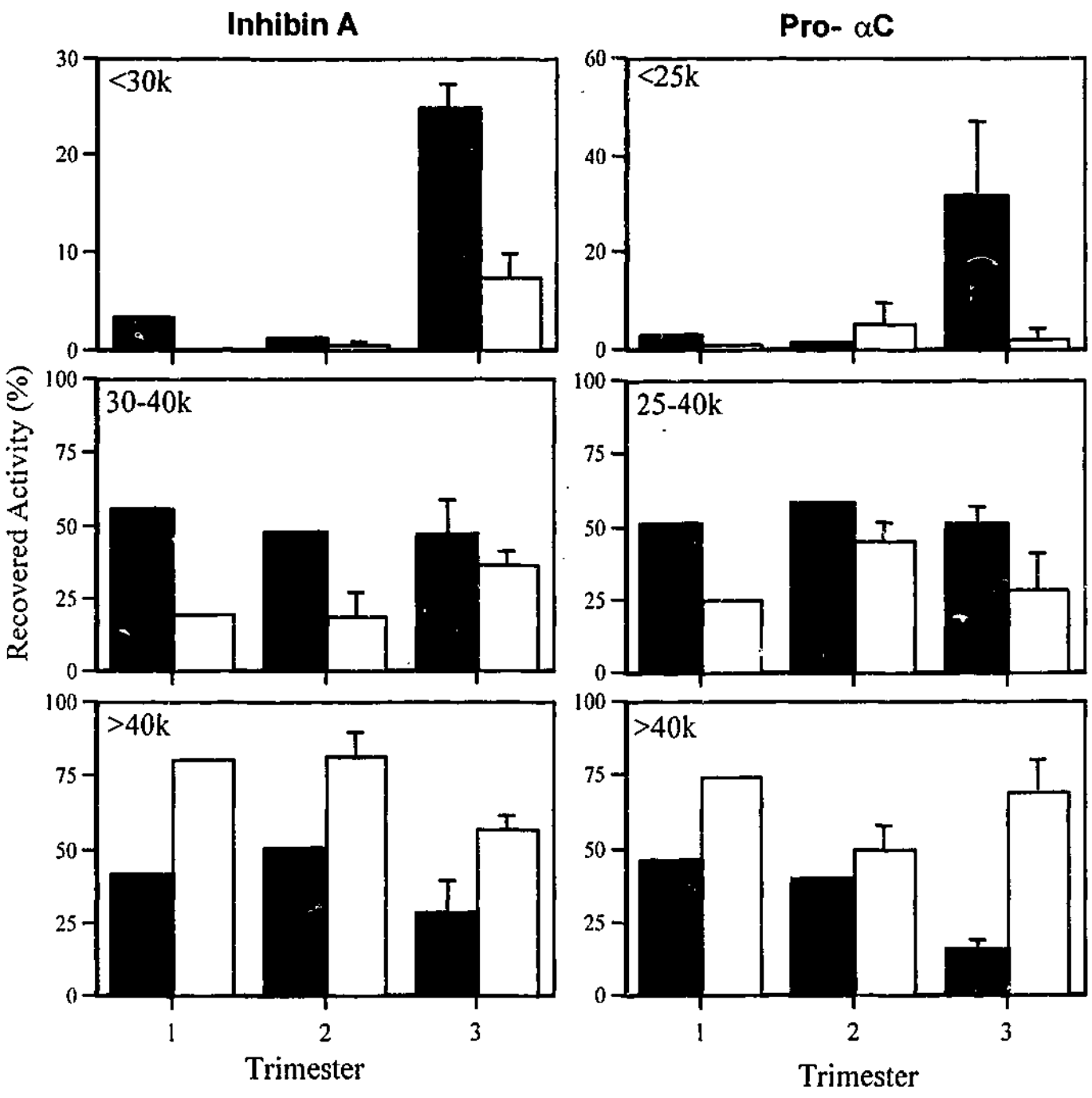



**Figure 5.2:** Changes in the proportion of recovered inhibin A and pro-αC in different mol wt regions throughout pregnancy. The data (% mean ± SD) represent results from maternal serum (closed bars) and placental extracts (open bars) in the three trimesters of pregnancy. The pools were fractionated through a combined IA/prep-PAGE procedure.

## 5.5 DISCUSSION

Using an improved immunofractionation procedure, Chapter Four demonstrated that the mol wt forms of inhibin in the maternal circulation alter with advancing gestation such that there is an increase in small mol wt forms of inhibin A and pro-αC after 19

weeks' gestation. Applying the same techniques to placental extracts in this study, it was explored whether those changes were due to altered placental secretion as the placenta contributes to the majority of inhibin detectable in maternal serum (Riley et al., 1996; Wallace et al., 1997a; Dalgliesh et al., 2001). The recoveries obtained here for fractionation of placental tissues are comparable with those obtained for pregnancy serum/plasma, presented in the previous chapter (Chapter Four), enabling direct comparisons of the proportions of molecular weight regions between serum and placentae. In addition, the biphasic profile of immunoinhibin levels, reported by several investigators (Abe et al., 1990; Tabei et al., 1991; reviewed by Qu and Thomas, 1995; Illingworth et al., 1996a; Fowler et al., 1998), where serum levels rise to a peak at 6-12 weeks gestation, fall at approximately 14 weeks and thereafter rise in the third trimester to another peak at term, is also observed in the placental profiles presented above, garnering further confidence in the fractionation system used in these studies.

In this study, first, second and term placental extracts contained key products of the diglycosylated form of the $\alpha\beta_A$-subunit (~36k), the monoglycosylated Pro-αC form and high mol wt forms of 55k, 66k, 75k and ~97k, corresponding to structures of Pro-αN-αC, Pro-αN-αC/$\beta_A$, αC/proβ-$\beta_A$ and αN-αC/proβ-$\beta_A$, respectively. Of these larger mol wt forms, only the 55k and 66k forms were identified in maternal serum (see Chapters Three and Four). This suggests that the placenta secretes highly glycosylated forms, leading to increased sizes of the inhibin α-subunit and the absence of these forms in serum is caused either by processing of the 97-75k forms to smaller 66-29k forms in serum or that the larger forms are not released into the circulation. The first possibility is more likely considering that a similar finding was reported when bovine 58k inhibin was cleaved to a 30k form when incubated in serum (McLachlan et al., 1986b). Interestingly, the proportion of 30-40k forms and 25-40k forms for inhibin A and pro-αC, respectively, in serum and placenta are quite similar and there were no changes in these proportions across trimesters, suggesting that if cleavage was occurring in the circulation it was not of these isoforms.

These results also suggest that the proβ-$\beta_A$ region is particularly susceptible to cleavage in serum, given that combinations with the full length $\beta_A$-subunit precursor is

not identified in maternal serum. The presence of low levels of small mol wt forms in the term placenta suggests cleavage of either the N terminal region, the C region of the α-subunit or the pro-sequence of the α subunit, most probably by placental furins (Nakayama, 1997). These combinations of the α and $\beta_A$ forms are all likely to be produced and secreted by the syncyntiotrophoblast as other investigators have localized the α and $\beta_A$ subunits in this placental cell type (Qu and Thomas, 1992; Minami et al., 1992).

Similar to the findings reported here, the inhibin mol wt forms reported in term placental extracts in previous studies (de Kretser et al., 1994; Muttukrishna et al., 1995) also showed that the majority of inhibin A immunoactivity was present in the ~32k range. However, those studies showed no evidence of smaller mol wt forms in placental tissue. This may be due to the limited resolution of the fractionation method - gel filtration - used in those studies which would not have readily detected the low proportions of small mol wt forms (7.3% and 2.2% for inhibin A and pro-αC, respectively) present in the term placenta.

While the proportion of small mol wt forms are indeed increased in term placental extracts compared to early pregnancy placental extracts, this increase does not mirror the abundance of these small forms observed in late pregnancy maternal serum (Chapter Four). This is particularly so for pro-αC. This suggests that altered placental secretion across pregnancy cannot completely account for the increase in small mol wt inhibin forms observed in maternal serum with advancing gestation and that other mechanisms, chiefly increased cleavage by a late pregnancy serum protease (discussed in Chapter Four), most likely underlie this observation. In keeping with this, the placenta contains more large inhibin A and pro-αC mol wt forms (>40k) and less small mol wt forms compared with maternal serum, suggesting that the small mol wt forms observed in serum could be due to increased cleavage in the circulation of only the large mol wt forms secreted by the placenta.

Another possible explanation for the differences in small mol wt forms between placenta and serum is that the fetal membranes may be an important source of circulating inhibins. It is now generally believed that inhibin A is primarily of

placental origin (Muttukrishna et al., 1995) while pro-αC is a product of the corpus luteum during early pregnancy (Illingworth et al., 1996a; Muttukrishna et al., 1997a; Lockwood et al., 1997) and the fetal-placental unit during late pregnancy (Riley et al., 2000). Of the two fetal membranes layers, the amnion selectively expresses β-subunit mRNA (Petraglia et al., 1993) which is more in keeping with activin production. The chorion however, expresses mRNA for both the α and β-subunits (Petraglia et al., 1993) consistent with inhibin secretion. Therefore, it is possible that small mol wt forms of inhibin are secreted by the chorion into the maternal circulation. However, it has been shown previously that the chorion most likely secretes primarily into the amniotic fluid rather than serum and that the chorion is an important source of inhibin B in that compartment (Wallace et al., 1998a; Petraglia et al., 1993). If the chorion also contributed significantly to circulating inhibins it might also be expected that inhibin B would be present in serum, which it is not (Wallace et al., 1997a, 2000; Riley et al., 2000). Furthermore, changes in chorionic inhibin secretion across pregnancy would be required to explain the gestation-related differences. While this is feasible, with advancing pregnancy the placenta becomes progressively larger relative to the volume of fetal membranes making an increasing contribution from the chorion less likely. Accordingly, a chorionic source is an unlikely explanation for the observations reported here, as is a fetal source. Only levels of inhibin B are reported in the cord serum from male fetuses, with neither inhibin A nor inhibin B present in cord serum from female fetuses (Wallace et al., 1997a; Wallace et al., 2000), making the fetus an unlikely source of the small mol wt forms.

In summary, the data presented here strongly suggests that the placenta is not solely responsible for the small mol wt forms of inhibin A and pro-αC observed in late pregnancy and, through a process of exclusion of other possible sources, it is postulated that increased cleavage occurs in the maternal circulation. Although levels of these small mol wt forms are low, they may be important under some physiological or clinical circumstances. The next two chapters examines whether these small mol wt forms have any clinical utility in certain maternal or fetal disease states.

# CHAPTER SIX

## Inhibins in Preeclamptic Pregnancies

## 6.1 SUMMARY

This study examined the different molecular weight forms of inhibin in normal and preeclamptic (PE) placental tissues and maternal serum using a combined immunoaffinity chromatography, preparative PAGE and electroelution procedure detailed in Chapter Three. Inhibin A and the pro-αC region of the α subunit were measured in the eluted fractions by specific ELISAs and the profiles of immunoactivity characterised in terms of mol wt and % recovery. The mol wt patterns of inhibin A and pro-αC in both PE and control placentae in late pregnancy showed peaks between 25-40k, 55k-75k and 97k, consistent with the presence of known mature and larger precursor inhibin forms, with greater abundance of the 25-40k forms in PE placentae and less small- (<30k) and large (>40k) inhibin A and pro-αC forms.

To assess whether these differences were reflected in the maternal circulation, serum from PE and control pregnancies in the third trimester (25-36 weeks' gestation) were processed and fractionated as above. Both groups showed inhibin A and pro-αC peaks between 25-40k and 55k-75k with a relative abundance of the 30-40k inhibin A and 25-40k pro-αC forms in PE serum. These results suggest that differences in the proportion of certain inhibin A and pro-αC molecular weight forms in PE placentae is due to trophoblast dysfunction with resulting impaired processing/cleavage of inhibin forms and subsequent secretion or spillage into maternal circulation.

## 6.2 INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific syndrome that is the principal cause of maternal morbidity and mortality, accounting for almost 15% of pregnancy-associated maternal deaths if the condition develops in early gestation (Roberts and Redman, 1993; American College of Obstetricians and Gynaecologists, 1996). The disease is typically characterised by hypertension and proteinuria in the second half of pregnancy.

As detailed in Chapter One (section 1.8.1), the precise pathogenesis of PE is yet to be elucidated but it is clear that the placenta is central to the disease which disappears soon after birth or after pregnancy termination (Palma Gamiz, 1998), when the placenta is no longer present. More specifically, several investigators have shown that impaired placentation is associated with PE, resulting in defective trophoblast invasion of the maternal spiral arterioles (Pijnenborg et al., 1980, 1981; Roberts and Redman, 1993; Ness and Roberts, 1996). This in turn is thought to lead to placental ischaemia and the release of, as yet unidentified, factors inducing maternal endothelial damage (Roberts et al., 1989). To date, several placental proteins have been studied as both candidate factors and as possible markers of PE, including free β-hCG (Raty et al., 1999; Lambert-Messerlian et al., 2000), corticotrophin-releasing factor (CRH) (Campbell et al., 1987; Ahmed et al., 2000), placental growth factor (PlGF) (Reuvekamp et al., 1999), renin (August et al., 1990; Matinlauri et al., 1995; Raty et al., 1999) and angiogenin (Kolben et al., 1997; Reuvekamp et al., 1999). Unfortunately, while all of these are indeed perturbed in association with PE, none have afforded adequate sensitivity and specificity to have any clinical utility as markers.

In this context, the placenta is also the main source of circulating inhibin A during human pregnancy (Illingworth et al., 1996a; Muttukrishna et al., 1997a) and many investigators have examined the biology of this glycoprotein in PE (Muttukrishna et al., 1997b; Cuckle et al., 1998; Aquilina et al., 1999; Silver et al., 1999; Gratacos et al., 2000; Muttukrishna et al., 2000). Maternal serum inhibin A has been shown to be 10-fold higher in women with established severe PE compared to gestational age-

matched controls (Muttukrishna et al., 1997b; Fraser et al., 1998). More importantly, levels of inhibin A were found to be elevated at 13-18 weeks in women who subsequently developed PE (Cuckle et al., 1998; Aquilina et al., 1999; Sebire et al., 2000), suggesting that inhibin may have a role in the pathogenesis of PE and that it may be a useful predictor of the disease. However, formal evaluations of the screening efficacy of dimeric inhibin A as a predictor of PE have been less promising (Muttukrishna et al., 2000; Lambert-Messerlian et al., 2000; Grobman and Wang, 2000). Whether improved discrimination between controls and PE cases might be achieved through the measurement of specific molecular weight forms of inhibin A, rather than 'total' inhibin A, has never been investigated.

As demonstrated in Chapter Five, inhibin A exists as a large number of molecular weight forms in the placenta, with these forms attributed to processing of the precursor subunits at recognized basic amino acid cleavage sites (Robertson et al., 1995). The principle aim of this study was to compare the inhibin molecular weight forms present in normal and PE placentae with the underlying hypothesis that the placental production of inhibin isoforms would differ between PE cases and controls and that this difference would be reflected in maternal serum, offering prospects of a better marker.

## 6.3 METHODS

### 6.3.1 Definition of preeclampsia

PE was diagnosed according to the criteria by the International Society for the Study of Hypertension in Pregnancy (ISSHP) and the Australian Society for the Study of Hypertension in Pregnancy (ASSHP) (Perry and Beevers, 1994; Brown et al., 2000):

- a sustained rise in blood pressure to greater than 140mmHg systolic or 90mmHg diastolic in a woman normotensive before the 20$^{th}$ week of pregnancy, and
- new and sustained proteinuria with a urinary excretion of at least 0.3g protein in a 24h specimen.

The studies had the approval of the Monash Medical Centre Human Research and Ethics Committee and informed consent was obtained from each patient.

### 6.3.2 Placentae

Placental tissues were collected within a few minutes of delivery and prepared as previously detailed in Chapter Five (section 5.3.2). Every effort was made to handle tissues from normal and PE pregnancies equivalently with respect to speed of handling and preparation.

Eight women with PE were delivered prior to labour by caesarean section, either pre-term (32-36 weeks' gestation) (n=6) or at term (n=2). There was no obvious intrauterine fetal growth restriction in any of the PE pregnancies, as defined by birth weight >10$^{th}$ percentile for gestation and hypertension and proteinuria in this group regressed after delivery. Adequate control groups, encompassing women delivering preterm with pregnancies not complicated by maternal or fetal pathologies, were difficult to acquire. Therefore, the control group in this study consisted of 3 healthy women with a normal, uncomplicated singleton pregnancy, delivered at term (37-40 weeks' gestation) after a spontaneous labour and normal vaginal delivery.

### 6.3.3 Maternal Serum

All aliquots of normal and PE maternal serum (25-36 weeks' gestation) used in this study came from samples collected between 1998-2000 from women attending the antenatal clinics of Monash Medical Centre. The women all had a singleton pregnancy. Gestation was determined from the last menstrual period or from a first trimester ultrasound scan. Bloods were collected in plain serum-gel tubes, stored at 4°C and centrifuged within 24h of collection. The serum was then stored frozen at –20°C.

Three control serum pools were prepared (25-36 weeks' gestation), with each pool consisting of equal aliquots from >20 women. Women in the control group were not

on medication and had no hypertension or proteinuria, nor subsequently developed hypertension or proteinuria.

Two PE serum pools were prepared (29-35 weeks' gestation). The first consisted of equal aliquots from 10 women and the second consisted of equal aliquots from 8 women. All pools were stored frozen at –20°C until processed.

### 6.3.4 Fractionation Procedure

Tissue and serum samples were fractionated as previously described and validated (Chapter Three, section 3.2.3). Samples were measured using modified inhibin A and pro-αC ELISAs, detailed and validated in Chapter Three (3.2.4).

### 6.3.5 Statistical Analyses

Data are presented as mean ± SD unless otherwise stated. The immunoactivity levels/fraction are also presented as a percentage of recovered activity (Figures 6.2-6.4). Differences in abundancies of mol wt forms between control and PE serum/placentae were assessed by a *t*-test. Significance was accorded when $p<0.05$.

## 6.4 RESULTS

### 6.4.1 Recoveries

Recoveries of inhibin A and pro-αC immunoactivities for the series of serum and placenta in the PE group are presented in Table 6.1 below. The combined IA and prep-PAGE recoveries are comparable with those obtained for control serum and placentae (25-35%, Chapters Four and Five, respectively).

**Table 6.1:** Recoveries (%) of inhibin A and pro-αC through the immunoaffinity (IA) and preparative-PAGE fractionation procedure of PE serum/placentae. n=number of fractionations, with 8-10 samples per fractionation.

| | Sample | n | IA | Prep-PAGE | Combined IA + Prep-PAGE |
|---|---|---|---|---|---|
| **Inhibin A** | Serum | 2 | 59.8 | 60.3 | 33.5 |
| | Placenta | 1 | 44.5 | 46.7 | 23.3 |
| **Pro-αC** | Serum | 2 | 53.4 | 55.7 | 26.7 |
| | Placenta | 1 | 52.2 | 61.1 | 28.9 |

### 6.4.2 Placenta

Inhibin B was undetectable in all placental extracts. Examples of typical mol wt profiles of inhibin A and pro-αC immunoactivity in control and PE placentae following fractionation are presented in figure 6.1. The inhibin A profile for normal placenta showed an abundance of large mol wt forms (97k, 75k and 66k). The PE placenta however, showed very little of these large mol wt forms (figure 6.1) but an abundance of the processed forms of inhibin A, primarily the mature 36k inhibin A form. No such differences were observed with pro-αC (figure 6.1).

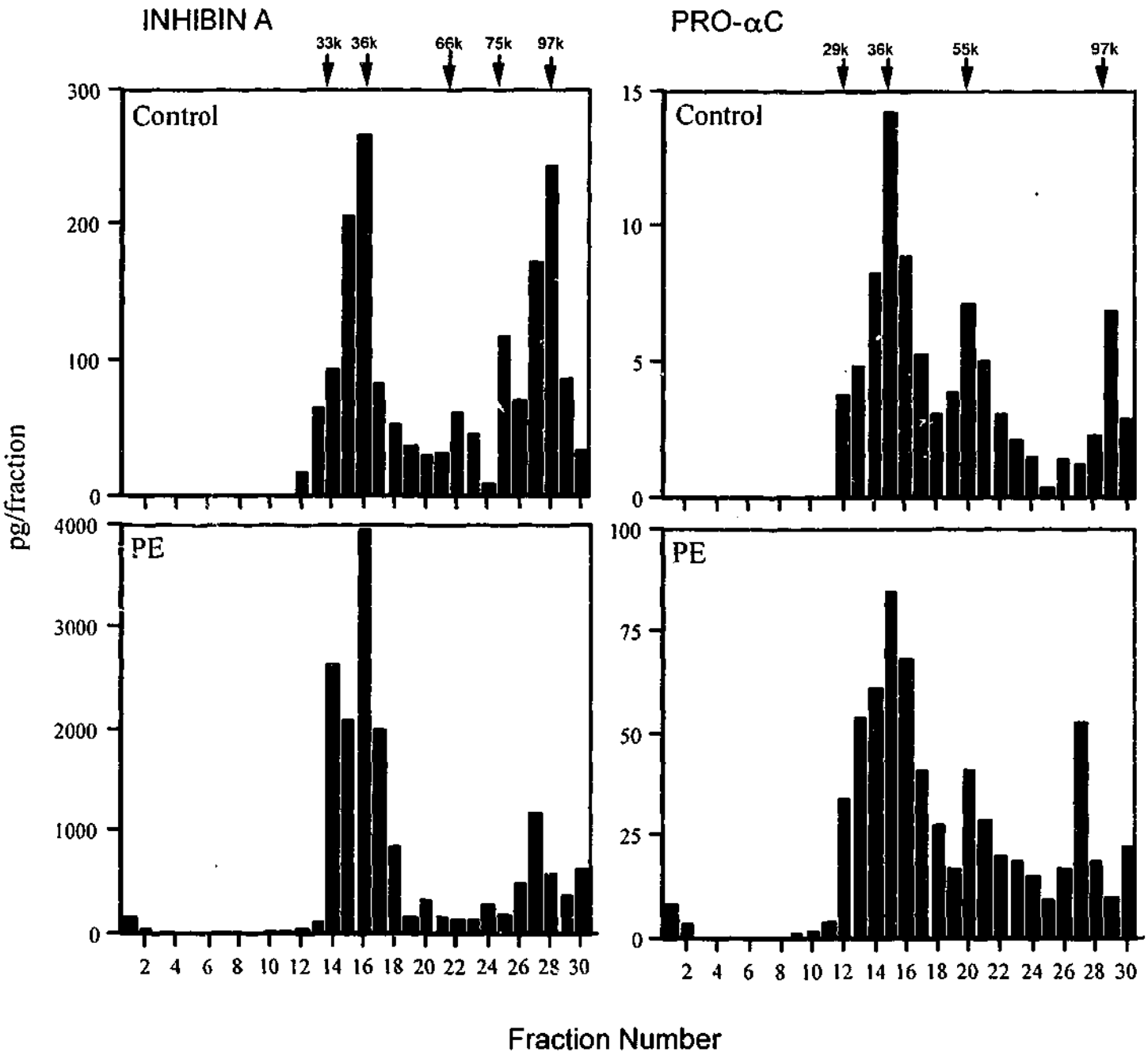



**Figure 6.1:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in normal and PE placental pools fractionated through a combined IA/prep-PAGE procedure.

The apparent differences of inhibin A between normal and PE placental extracts are presented in figure 6.2, where changes in 3 mol wt regions are highlighted for inhibin A (<30k, 30-40k and >40k) and pro-αC (<25k, 25-40, and >40k). More 30-40k inhibin A forms were present in PE placentae than normal (52.6% vs 36.2% respectively) with less small (<30k) (2.4% vs 7.3%) and large (>40k) forms (46.4% vs 56.5%) in PE placenta than in the normal (figure 6.2). Similar patterns were

demonstrated with pro-αC, with more 25-40k forms in PE placenta compared with normal (42.7% vs 28.4%) and less small (<25k) (0.9% vs 2.2%) and large (>40k) (69.4% vs 56.4%) PE pro-αC forms than controls (figure 6.2).

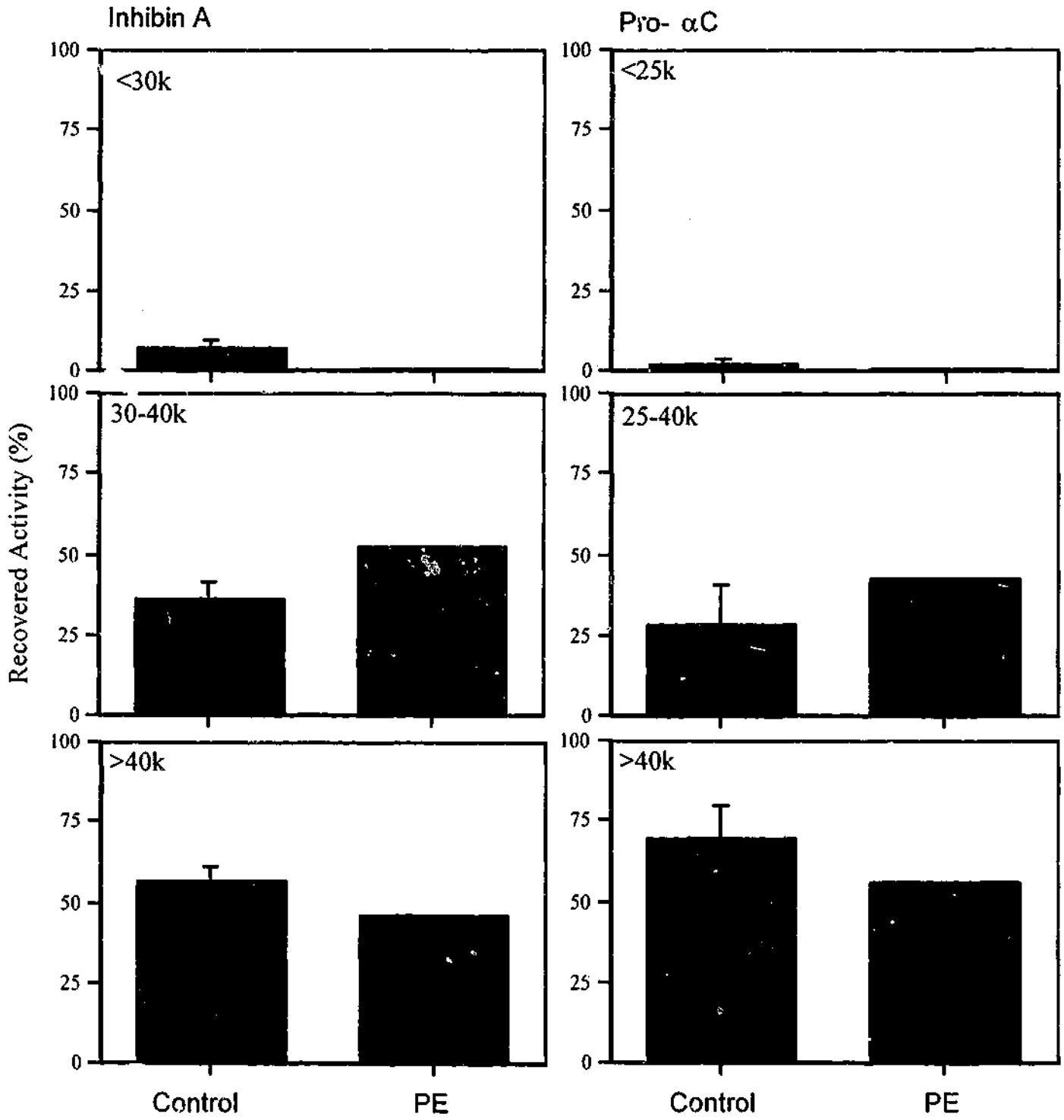



**Figure 6.2:** Changes in the proportions of recovered inhibin A and pro-αC in different molecular weight regions in normal and PE placentae.

### 6.4.3 Maternal Serum

Examples of typical mol wt profiles of inhibin A and pro-αC immunoactivity in PE and control maternal serum following fractionation are presented in figure 6.3. Inhibin B was undetectable in all serum samples. The profiles are similar for both normal and PE pregnancies, with primarily the mature 31-36k inhibin A and 27-36k pro-αC forms present (Figure 6.3).

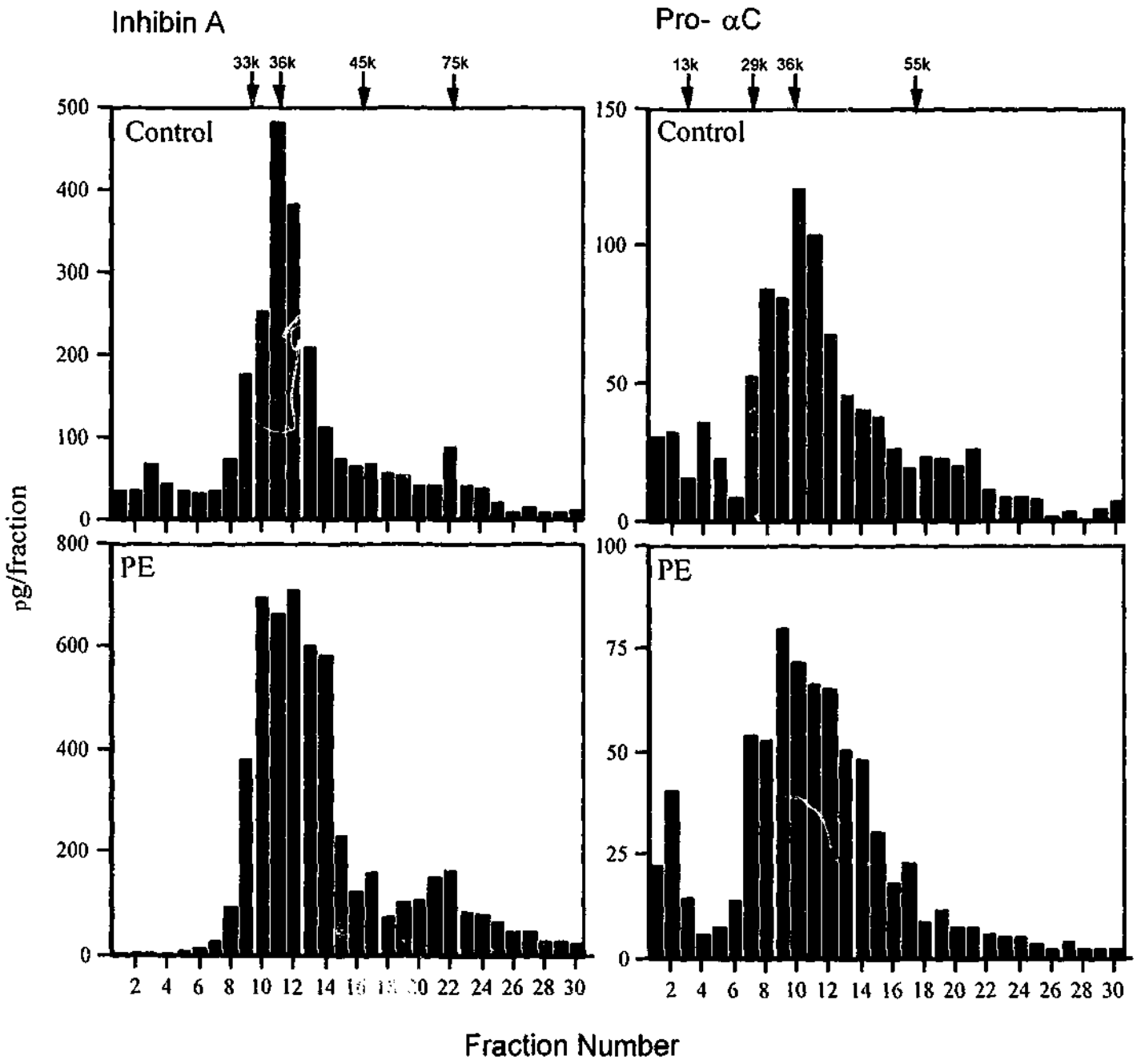



**Figure 6.3:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in control and PE serum pools fractionated through a combined IA/prep-PAGE procedure.

To compare between chromatograms, the inhibin A and pro-αC patterns were divided into regions based on mol wts of the peak regions and the immunoactivity values calculated as a percentage of recovered activity (Figure 6.4). PE serum profile showed a greater abundance of inhibin A 30-40k forms compared to controls (60.9% vs 38.7%, respectively; p=0.02) with little difference in the abundance of the <30k and >40k forms (p=0.08) (figure 6.4). Similarly, the PE serum profile showed a minimal, but significant, increase of pro-αC 25-40k forms compared to controls (64.5% vs 54.2% respectively; p=0.045) and significantly less <25k forms (8.6% vs 34.3%, p=0.003) with little difference in the abundance of the >40k forms (p>0.05) (figure 6.4).

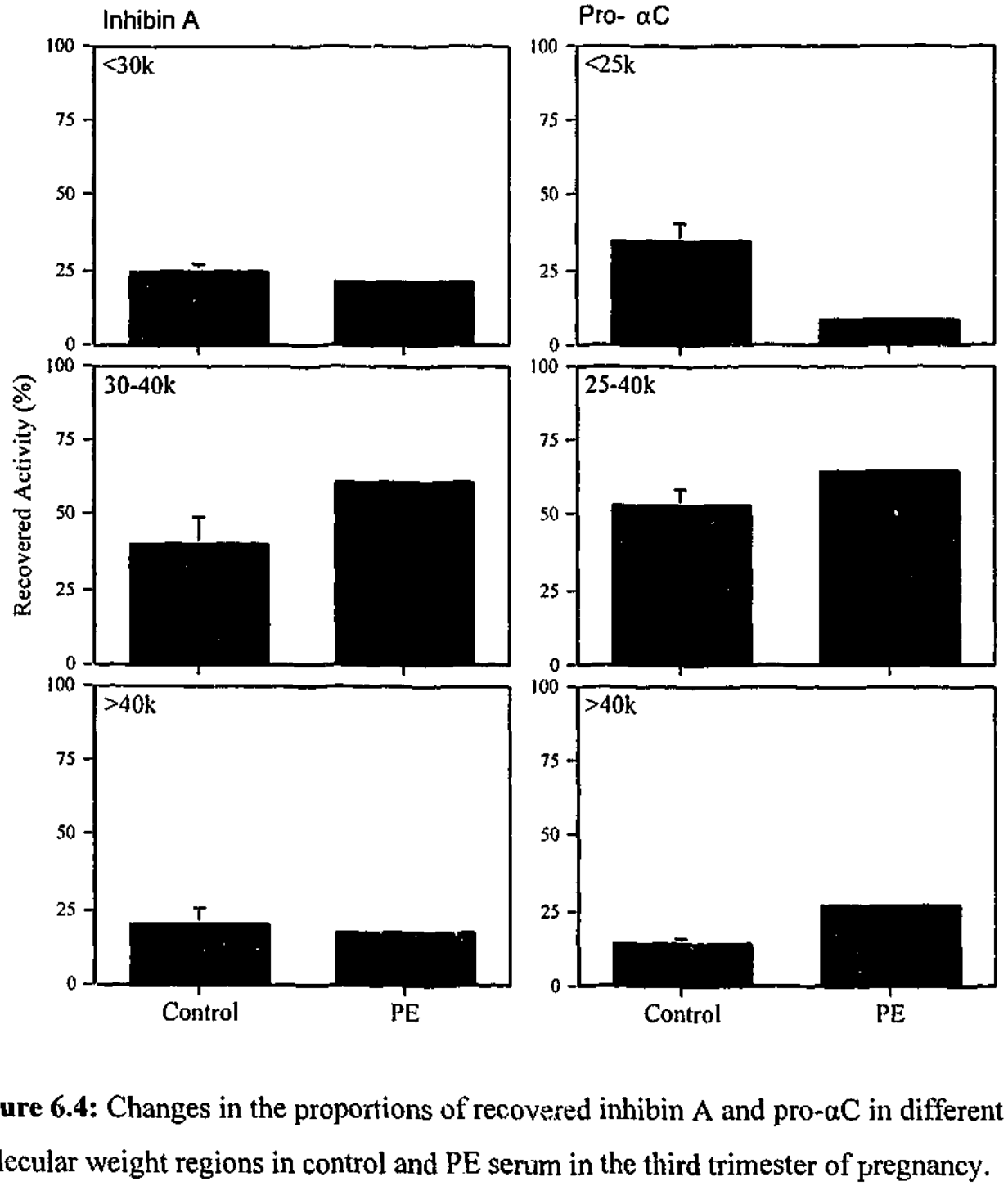



**Figure 6.4:** Changes in the proportions of recovered inhibin A and pro-αC in different molecular weight regions in control and PE serum in the third trimester of pregnancy.

## 6.5 DISCUSSION

As discussed in previous chapters, it is well established that inhibins exist as multiple molecular weight forms and more specifically, that the proportions of these forms in maternal serum alters with gestation. This study was undertaken to determine if the molecular weight forms of inhibin A and pro-αC in PE placentae and serum differed from that in normal pregnancies. As the trophoblast is the source of inhibin in pregnancy, alterations in their production are not unexpected given the pivotal role of the placenta in PE (Ness and Roberts, 1996; Dekker and Sibai, 1998) and the existing literature showing increased levels of inhibin A in association with PE (Muttukrishna et al., 1997b; Cuckle et al., 1998). The results obtained demonstrate that PE placentae contain several inhibin A and pro-αC molecular weight forms and these forms are *not* substantially different from those present in normal pregnancy.

In this study, both PE and normal placentae were shown to contain inhibin A mol wt peaks of 97k, 75k, 66k and 36k, identical to the forms demonstrated for 1st, 2nd and term placentae in Chapter Five. As discussed in Chapter Five, these forms most probably correspond to the known fully- and partially processed inhibin forms of αN-αC/proβ-$\beta_A$, αC/proβ-$\beta_A$, pro-αN-αC/$\beta_A$ and αC/$\beta_A$ (Robertson et al., 1995, 1996, 1997a). However, although there were no differences in the *types* of forms present, there were significant differences in the relative *abundancies* of these forms between PE and normal placentae. The PE placentae contained less of the large 97-75k forms and more of the mature 36k form. These differences are unlikely to be due to gestational differences between the tissue groups as there was little difference in the abundance of these molecular weight forms between second trimester and term tissues (Chapter Five). Whether the differences between the PE and normal group result because of differences in the mode of delivery (labouring in normal and caesarean section in PE) or reflect abnormal production/cleavage due to trophoblast dysfunction is difficult to distinguish. It has been suggested that the placenta undergoes tissue deterioration during labour and vaginal delivery, disrupting endoproteolytic enzymes compartmentalized in trophoblast (Ahmed et al., 2000). This provides opportunity for protease activation *in vitro*, resulting in increased protein processing (Ahmed et al.,

2000). This is unlikely to underlie the differences in inhibin mol wt forms observed in this study as the reverse is observed: PE placentae (obtained after caesarean delivery) contained more processed inhibins, with a lesser abundance of large mol wt forms, than the normal placentae, collected after labour. Likewise, as every effort was made to handle tissues from normal and PE pregnancies equivalently with respect to speed of handling and processing, it is unlikely that the differences observed between groups were the result of differential preparation. Therefore, it is more likely that the differences in the abundancies of large inhibin A molecular weight forms between the groups are a result of abnormal cleavage due to degeneration within the trophoblast, attributed to changes associated with PE.

These differences in abundancies were also present between normal and PE pro-αC mol wt forms, although to a lesser extent than for inhibin A. As it is thought that trophoblast, in particular syncytiotrophoblast, synthesizes the inhibin α- and β subunits (McLachlan et al., 1986a; Petraglia et al., 1991; de Kretser et al., 1994; Qu and Thomas, 1995), this suggests that the differences in proportions of molecular weight forms observed in PE placentae are the result of altered placental function. Indeed, a recent study by Jackson et al (2000) reported a greater intensity of inhibin $\beta_A$ and α-subunit staining in the syncytiotrophoblast of PE placentae than controls. This altered placental function would most probably lead to altered regulation and/or cleavage of inhibin subunits in the placenta, with preferential secretion of small mol wt inhibins. Support for this comes from a recent study by Ahmed et al (2000), who reported an abundance of the corticotropin-releasing hormome (CRH)1-41 form in PE placental tissues, a small molecular weight form arising as a cleavage product of pro-CRH. This suggests increased placental production of small mol wt forms or abnormal cleavage of peptides associated with the pathophysiology of PE.

The finding of increased placental total inhibin A in PE in this study could account for the increased serum inhibin A levels in PE. The mature 33-36k inhibin A and 29-36k pro-αC forms, consisting primarily of the mono- and diglycosylated mature α-$\beta_A$ dimer and pro-αC respectively, were the pre-dominant circulating forms in both PE and control serum in this study, although, as with the case in placentae, these forms

were present in greater quantities in PE maternal serum than controls, with the difference being greater for inhibin A. This is perhaps not surprising as it is well established that concentrations of inhibin A ($\alpha$-$\beta_A$ dimer) are higher in the maternal serum of women with severe PE compared to gestational age-matched controls (Muttukrishna et al., 1997b; Fraser et al., 1998; Cuckle et al., 1998). The increase in inhibin A levels in maternal serum are interpreted as further evidence for trophoblast dysfunction in PE, with altered synthesis/secretion of the $\beta_A$ subunit into maternal circulation.

In conclusion, this study demonstrates that particular molecular weight forms of inhibin A or pro-$\alpha$C are unlikely to be better markers of PE than 'total' inhibin A. However, studies determining the proportions of particular inhibin molecular weight forms in maternal serum preceding the development of PE would be necessary to confirm this. Unfortunately, sufficient pre-disease serum was not available in the quantities required for immunoaffinity fractionation chromatography.

# CHAPTER SEVEN

## Inhibin Molecular Weight Forms in Aneuploid Pregnancies

## 7.1 SUMMARY

Second trimester maternal serum (MS), placental extracts and amniotic fluid (AF) pools from normal and aneuploid pregnancies were fractionated by the combined immunoaffinity chromatography, preparative PAGE and electroelution procedure detailed in Chapter Three. Inhibins A, B and pro-αC were determined in the eluted fractions by specific ELISAs and the profiles of immunoactivity characterised in terms of mol wt and percentage recovery. There were no differences in the mol wt patterns of inhibin A and pro-αC in MS and AF between trisomic and control pregnancies, with peaks between 25-40k and ~66k consistent with the presence of known mature and larger precursor inhibin forms. However, AF contained relatively more small molecular weight forms (<30k inhibins A and B and <25k pro-αC forms), consistent with different sources of inhibins in the different pregnancy compartments. There were large differences between the forms present in control and DS placental tissues, with control tissues showing inhibin A and pro-αC peaks of ~97k and 25-40k. In contrast, the Down syndrome (DS) placenta showed little evidence of the large mol wt forms but an abundance of the 25-40k forms. This suggests abnormal processing, cleavage or secretion of inhibin mol wt forms by the DS placenta. These differences were not reflected in MS, suggesting further proteolysis of large inhibin mol wt forms in circulation. It is concluded that while the DS placenta produces different abundances of the mol wt forms of inhibins than the normal placenta, these differences were not apparent in serum and are therefore not of clinical utility.

## 7.2 INTRODUCTION

Initial studies of inhibins in Down syndrome (DS) used inhibin assays that measured both dimeric inhibin and free inhibin α-subunits (immunoreactive (ir) inhibin). The results from these studies suggested that maternal serum levels of ir-inhibin were, on average, elevated in pregnancies with a Down syndrome (DS) fetus (Van Lith et al., 1992; Spencer et al., 1993). However, the degree of discrimination between DS and unaffected pregnancies afforded by ir-inhibin was not sufficient for it to be a clinically useful serum marker (Spencer et al., 1993; Cuckle et al., 1994b). The possibility that the degree of discrimination might depend on the specificity of the inhibin assay was first suggested by Wallace and colleagues in 1994 when, after measuring ir-inhibin in first trimester maternal serum from 11 DS pregnancies and 89 controls using two different immunoassays, they found a poor correlation between the two, with the results derived from one assay failing to discriminate between the DS and control pregnancies.

The subsequent development and application of a sensitive and specific immunoassay for inhibin A (Groome and O'Brien, 1993) confirmed these earlier suggestions by Wallace et al (1994), with several investigators showing that inhibin A was significantly more elevated than ir-inhibin in the same DS samples (Cuckle et al., 1994; Cuckle et al., 1995; Wallace et al., 1995). It is now well established that inhibin A is significantly elevated in DS samples in both the first and second trimesters of pregnancy (Cuckle et al., 1995; Wallace et al., 1996; Lambert-Messerlian et al., 1996; Noble et al., 1997). Indeed, inhibin A increases the prenatal detection rate of DS by up to 20% when added to existing prenatal serum markers (Aitken et al., 1996; Cuckle et al., 1996). Importantly, these studies measured 'total' inhibin A. The potential for particular molecular weight forms of inhibin A to be useful prenatal markers of DS has been largely ignored. As detailed in Chapter Four, inhibin A itself exists as a number of molecular weight forms in pregnancy serum and these forms alter substantially with advancing gestation. Therefore, the principal aim of this study was to compare the mol wt forms of second trimester MS inhibins, particularly inhibin A, between normal and DS pregnancies, exploring whether particular mol wt forms offer the possibility of even greater discrimination between cases and controls than total inhibin A.

In contrast to MS, levels of inhibin A and pro-αC in the amniotic fluid (AF) of DS pregnancies are significantly lower than normal (Wallace et al., 1997b, 1998a, 1999), suggesting that the regulation, secretion and/or metabolism of the inhibin subunits into this compartment is disturbed in abnormal pregnancies. Indeed, it was revealed that there were several different sites of secretion of inhibins A, B and pro-αC, with specific patterns of secretion in the AF and maternal circulation (Wallace et al., 1997a; Riley et al., 2000). Therefore, a second aim of this study was to compare the inhibin molecular weight forms present in the different compartments of normal and aneuploid pregnancy.

## 7.3 METHODS

All the studies detailed in this chapter had the approval of the Human Research and Ethics Committee of Monash Medical Centre and all patients gave informed consent.

### 7.3.1 Samples

#### *7.3.1.1 Maternal serum (MS)*

Aliquots of control and DS 2$^{nd}$ trimester MS (13-18 weeks' gestation) were obtained from prospective DS and neural tube defects prenatal screening programmes at Monash Medical Centre (Victoria, Australia) and The Women and Infants Hospital (Rhode Island, USA, kindly provided by Drs Geralyn Lambert-Messerlian and Jacob Canick) between 1998-2000. Gestation was determined from the date of the last menstrual period or from a first trimester ultrasound scan, when available. Bloods were collected in plain serum-gel tubes, stored at 4°C and centrifuged within 24h of collection. The serum was then stored frozen at –20°C until assay.

Two DS serum pools were prepared. The first consisted of equal aliquots from 10 women attending Monash Medical Centre and the second consisted of equal aliquots from 13 women attending The Women and Infants Hospital, Rhode Island. Similarly, two control serum pools were prepared from the respective centers. The first control pool consisted of equal aliquots from >28 women, prepared from women attending Monash Medical Centre and the second consisted of equal aliquots from 26 women, prepared from women attending The Women and Infants Hospital. Sera from both

control pools were chosen to match with the DS samples from the respective centres for completed week of gestation and duration of storage.

### *7.3.1.2 Amniotic Fluid (AF)*

AF was also collected prospectively at Monash Medical Centre as a component of the prenatal diagnostic service and stored at –20°C. All AF samples were separated for storage from fetal epithelial squamous cells by centrifugation at 250x*g* within 24 hours of collection, and an aliquot of each sample was stored specifically for this study at –20°C until the day of pooling. The karyotype from each sample was recorded. All amniotic fluid samples were derived from 2$^{nd}$ trimester pregnancies (15-18 weeks' gestation) on which an amniocentesis had been performed between 1998-2000.

Pools of AF, each of 30ml volume, were prepared from two separate groups. Group one consisted of AF from chromosomally normal pregnancies from which 3 separate pools were prepared from a total of 57 samples. Of these 57 amniocenteses, 78.9% (n=45) were performed for reasons of maternal age or maternal anxiety and 21.1% (n=12) because of an increased risk of aneuploidy, as determined by maternal serum screening.

Group two consisted of AF from aneuploid pregnancies. One AF pool was prepared from 12 DS samples, one pool from 6 trisomy 13 samples and one pool from 8 trisomy 18 samples. For these 36 samples, the amniocentesis had been performed for reasons of maternal age in 18% (n=7) of cases, for an abnormal ultrasound scan in 59% (n=23) of cases and for abnormal maternal serum screening in 23% (n=9) of cases. All AF pools were stored at –20°C until fractionated.

### *7.3.1.3 Placentae*

Placentae from 3 DS singleton pregnancies in the second trimester (15-18 weeks' gestation) were collected from otherwise normal healthy women undergoing surgical termination of pregnancy (dilatation and evacuation) for the aneuploidy. Intravaginal pre-treatment with 400µg misoprostil (Searle Pharmaceuticals, Crows Nest, NSW) was administered 2-3 hours before surgery, which was carried out under general anaesthesia.

Control tissues from 11 2nd trimester (15-18 weeks' gestation) singleton pregnancies were collected from normal healthy women with a normal pregnancy undergoing surgical termination of pregnancy (D&E) for psychosocial reasons. All tissues were prepared immediately as previously described (Chapter Five, section 5.3.2).

#### *7.3.1.4 Matched MS and AF (Normal)*

In 33 women, venepuncture was performed immediately prior to amniocentesis between 14-18 weeks of pregnancy. Cases where bloods were drawn after an invasive procedure were eliminated from this study as these could contain fetal cells, solely as a result of an iatrogenic feto-maternal haemorrhage (Elias and Simpson, 1994).

In 20 (61%) of these women, amniocentesis was performed for maternal age with the remainder (39%) performed because of an increased risk of aneuploidy by maternal serum screening. All cases were reported to have normal karyotype.

The blood was centrifuged and the serum separated on the day of collection and stored at –20°C until fractionated. The amniotic fluid was processed as detailed above. One pool each (30ml volume) of AF and MS was prepared from these 33 samples.

### 7.3.2 Fractionation Procedure

All samples were fractionated as previously described and validated (see Chapter Three, section 3.2.3).

### 7.3.3 Inhibin Assays

Inhibins A, B and pro-αC were measured using specific ELISAs described and validated previously (Chapter Three, section 3.2.4).

### 7.3.4 Data Analysis

Inhibin A, inhibin B and pro-αC chromatograms were divided into regions based on mol wts of the peak regions and the immunoactivity values calculated as a percentage of recovered activity. These percentages were compared between DS and control groups using *t*-tests and between different pregnancy compartments using paired *t*-

tests (Prism Package, GraphPad Software Inc., San Diego, CA). Statistical differences were considered to be significant when $P<0.05$.

## 7.4 RESULTS

### 7.4.1 Recoveries

Recoveries of inhibin A for the series of abnormal MS, AF and placentae are presented in Table 7.1. These recoveries are comparable with those obtained previously for normal maternal serum/plasma or placentae (combined IA + Prep-PAGE recoveries of 25-35%, Chapter Four (section 4.3) and Chapter Five (section 5.4.1), respectively.

**Table 7.1:** Recoveries (%) of inhibin A through the immunoaffinity (IA) and preparative PAGE fractionation procedure from DS maternal serum and placentae and aneuploid (DS, trisomy 18 and trisomy 13) amniotic fluid.

| Samples | n | IA | Prep-PAGE | Combined IA + Prep-PAGE |
|---|---|---|---|---|
| Maternal Serum | 2 | 58.6 | 52.3 | 27.7 |
| Amniotic Fluid | 3 | 59.4 ± 7.8 | 48.6 ± 10.2 | 29.3 ± 7.5 |
| Placentae | 1 | 53.4 | 54.3 | 25.6 |

### 7.4.2 Maternal Serum Profiles

An example of the molecular weight profile of inhibin A and pro-αC immunoactivity in control and DS MS following fractionation is presented in figure 7.1 below.

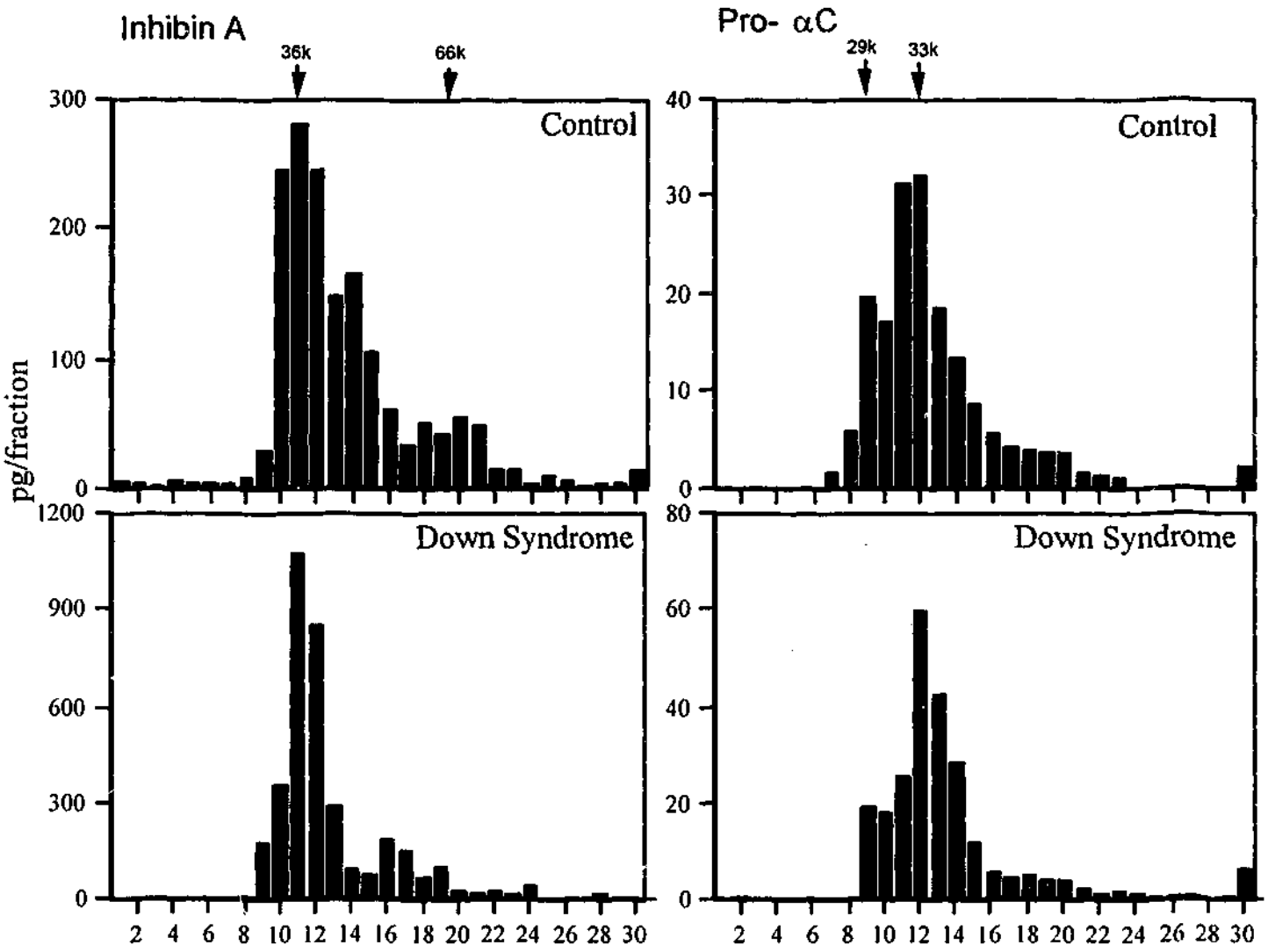



**Figure 7.1:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in MS control and DS pools fractionated through a combined IA/prep-PAGE procedure. Levels below the sensitivity of the assays were not included.

The control serum profiles were similar to the three 2nd trimester normal MS profiles presented in Chapter Four (13-18 week pool, 12-15 week serum pool and matched plasma pool, section 4.4) thus the data from these were pooled to give a more robust control serum profile. Control serum consisted primarily of the mature 32-36k and 66k inhibin A and 27-36k pro-αC forms, corresponding to known mature structures. The DS profile was similar to the control serum profile, with the same peaks identified (Figure 7.1). Inhibin B was undetectable in all serum samples.

To compare between chromatograms, the inhibin A and pro-αC patterns were divided into regions based on mol wts of the peak regions and the immunoactivity values

calculated as a percentage of recovered activity (a method used previously in Chapters Four and Five). There were no differences in inhibin A forms with mol wts <30k and pro-αC forms <25k (representing mol wts less than those recognised for recombinant inhibin A and pro-αC) in normal MS compared with DS MS (figure 7.2).

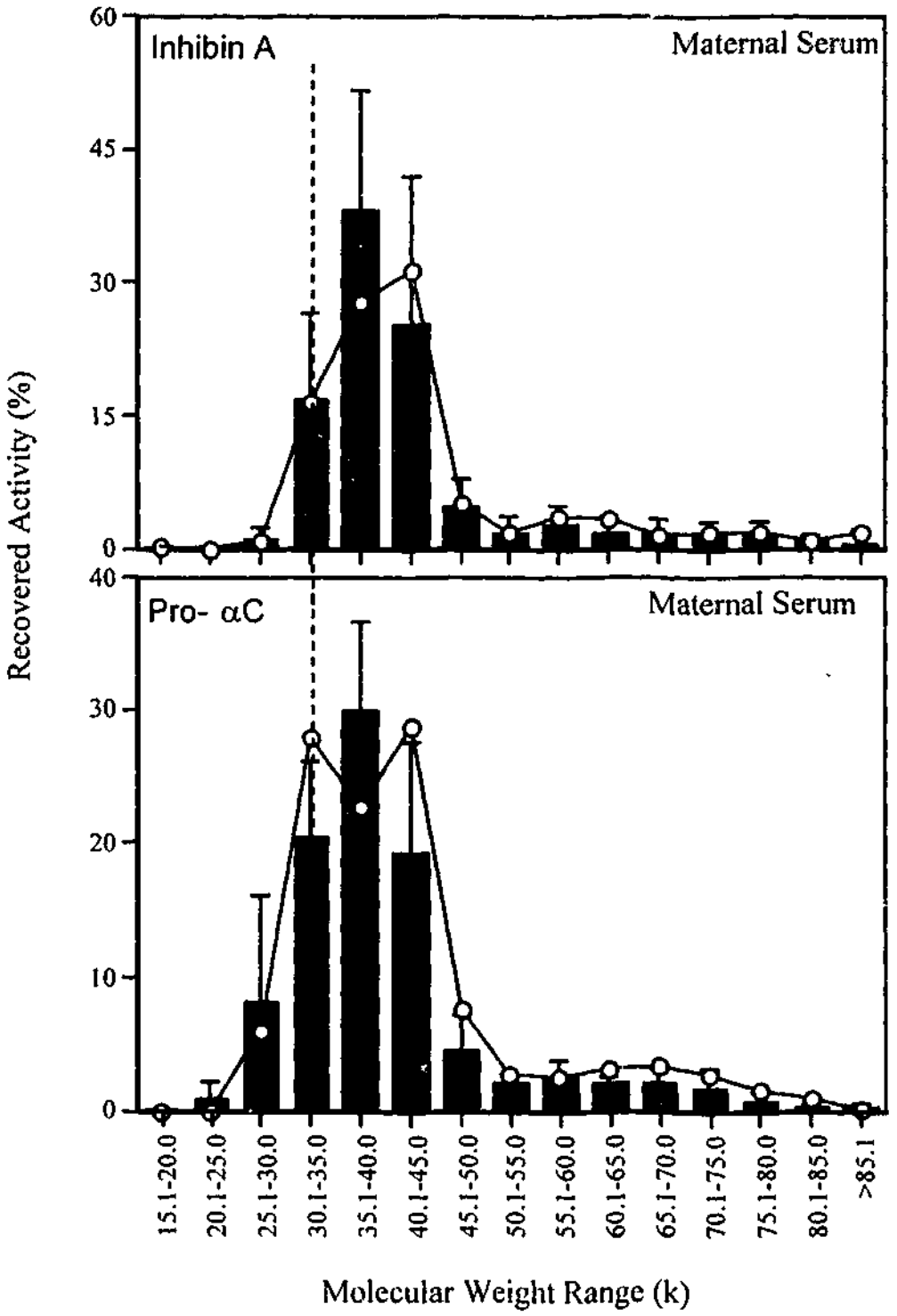


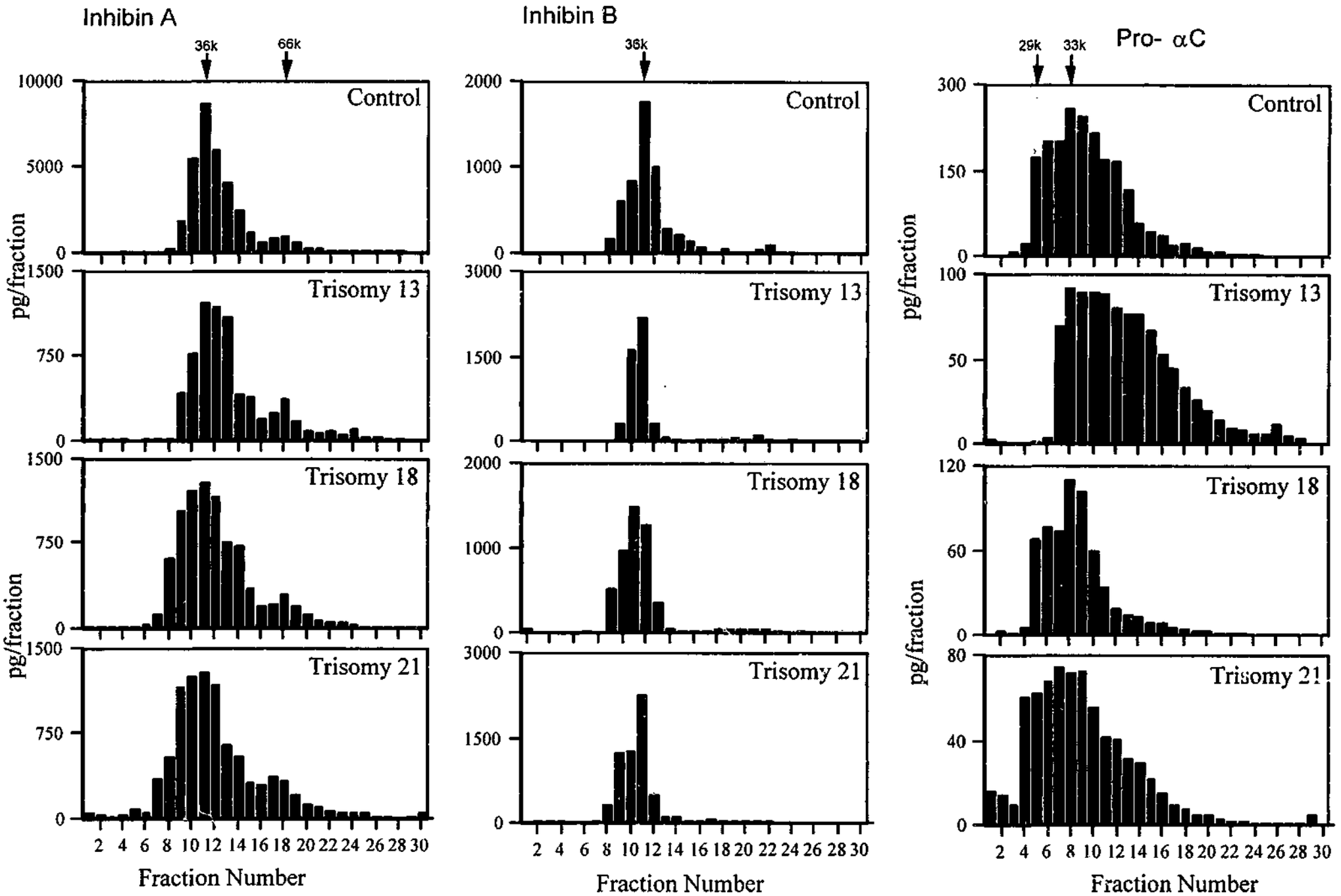

**Figure 7.2:** Mean ± SD mol weight distribution of inhibin A and pro-αC immunoactivities in MS control (closed bars) and DS (open circles) samples processed through the combined IA/prep-PAGE procedure. The profile is divided up into mol wt ranges for averaging purposes. The vertical dashed line denotes the mol wt of 31k inhibin A used as a reference.

### 7.4.3 Amniotic Fluid Profiles

Sexing information was available from all control and aneuploid AF. Thirty-five (61.4%) of the 57 control AF samples were from pregnancies with a female fetus. Of the aneuploid samples, pregnancies with a female fetus were lower: eight (32%) with DS, three (37.5%) with trisomy 18 and three (50%) with trisomy 13.

The levels of inhibin A and pro-αC in the aneuploid AF were significantly lower than control ($p<0.001$, inhibin A control vs trisomy 13, 18 and 21; $p<0.03$ pro-αC controls vs the three aneuploidies (figure 7.3). No significant differences in inhibin B levels were observed between control and abnormal AF ($p>0.05$ for all). Examples of the molecular weight profiles of inhibin A, inhibin B and pro-αC immunoactivity (figure 7.3) in control and aneuploid AF following fractionation are presented on the opposite page.

**Figure 7.3:** Molecular weight distribution of inhibin A, inhibin B and pro-αC immunoactivities in AF control and trisomic pools fractionated through a combined IA/prep-PAGE procedure.


Inhibin A
Inhibin B
Pro- αC
36k
66k
29k
33k
Control
Trisomy 13
Trisomy 18
Trisomy 21
pg/fraction
Fraction Number

The inhibin A, B and pro-αC molecular weight profiles were similar for control and all three aneuploidies, with primarily the 36k and 66k inhibin A, 36k inhibin B and 27-40k pro-αC forms present (figure 7.3), consistent with partially processed and processed forms of inhibin A, inhibin B and inhibin α subunit containing the pro-αC fragments (Robertson et al., 1996, 1997a).

When the small molecular weight forms of inhibin A and pro-αC in control and aneuploid AF were calculated, there were no differences in the proportion of <30k inhibin A and <25k pro-αC forms (figure 7.4) compared to control. However there were large differences in the <30k inhibin B forms between control and DS AF (0.7% vs 26.9%, respectively, p=0.01). There were no significant differences in the abundancies of the other regions between control and abnormal groups (figure 7.4).

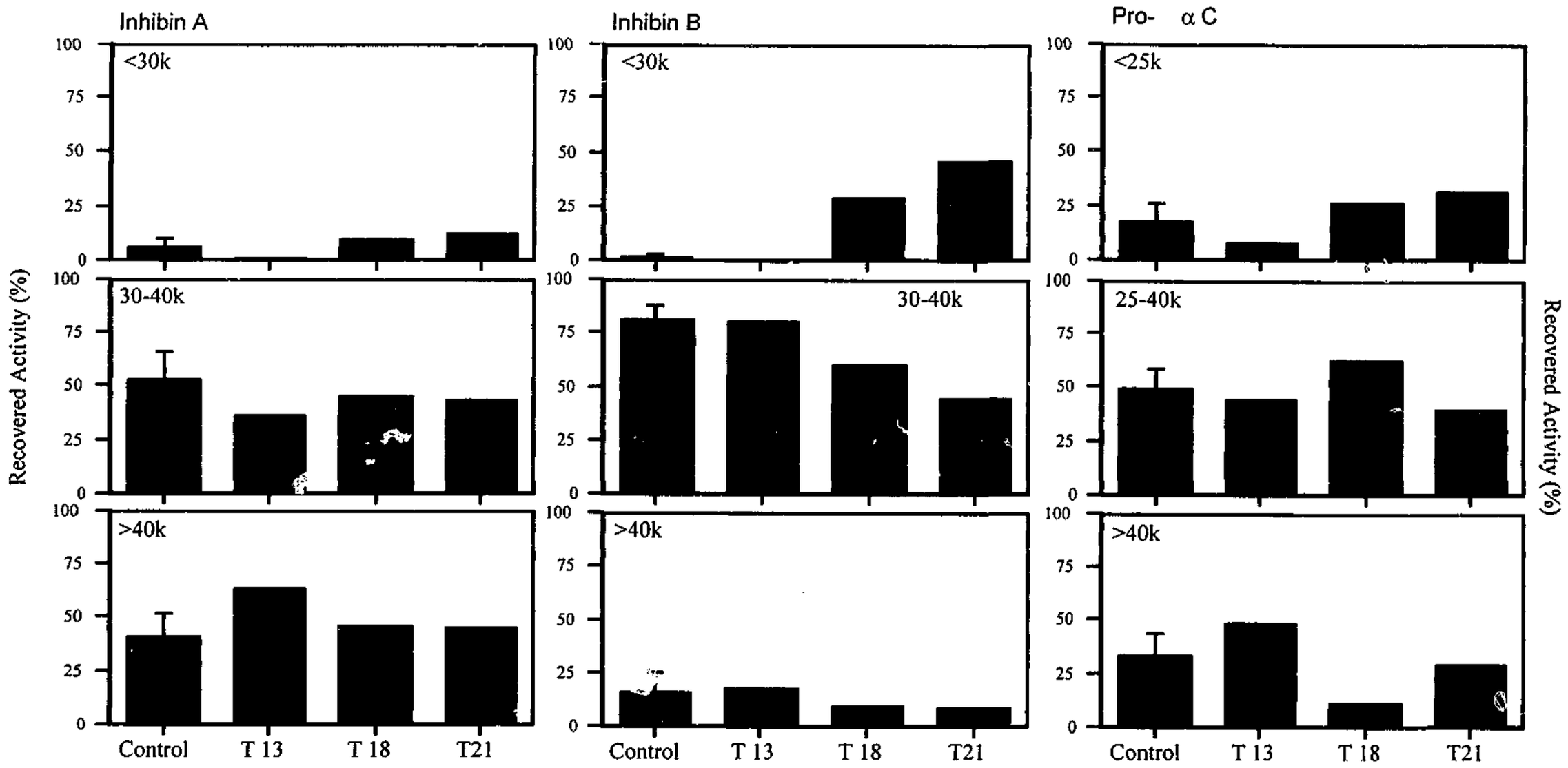



**Figure 7.4:** The proportions of recovered inhibins A, B (open bars) and pro-αC in different mol wt regions of normal and aneuploid AF pools (T13=trisomy 13, T18=trisomy 18 and T21=Down syndrome). The pools were fractionated through the combined IA/prep-PAGE procedure.

### 7.4.4 Placental Profiles

Inhibin B was undetectable in all placental lysates. The inhibin A and pro-αC DS profiles were compared with that obtained for normal 2nd trimester placentae, previously presented in Chapter Five (section 5.4.2). There were obvious differences in the molecular weight profiles of inhibin A immunoactivity between normal and DS placental extracts (figure 7.5).

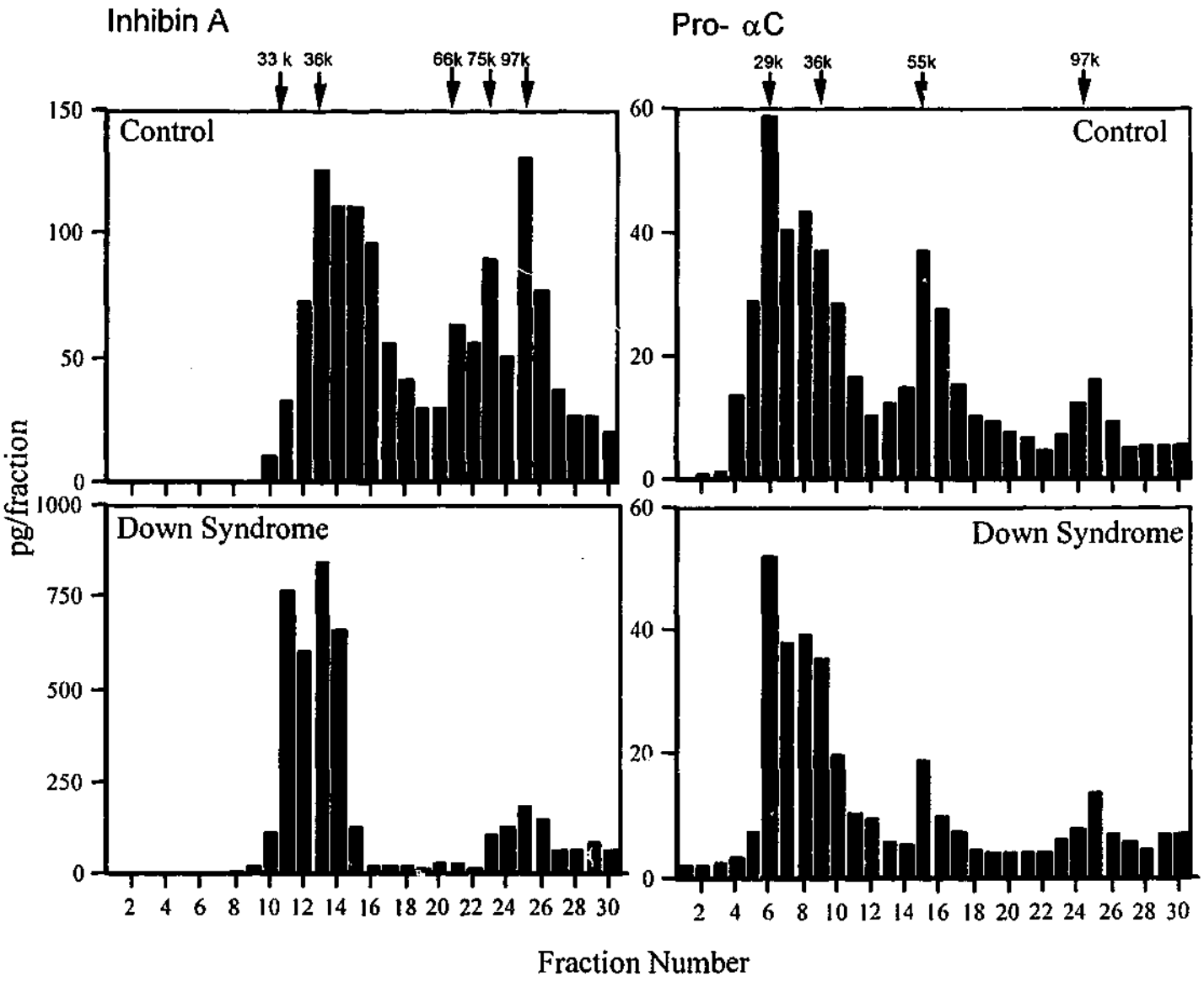



**Figure 7.5:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in placental extracts of control and DS pools fractionated through a combined IA/prep-PAGE procedure. Levels below the sensitivity of the assays were not included.

As previously described in Chapter Five, the inhibin A profile for normal placenta showed an abundance of large mol wt forms (~97k to ~66k) (figure 7.5), consistent with partially processed forms of inhibin A and inhibin α subunit containing the pro-

αC fragments. The DS placenta however, showed very little of these large mol wt forms (figure 7.5) but an abundance of the processed forms of inhibin A, primarily the mature 36k inhibin A form. These apparent differences in inhibin A between normal and DS placental extracts are presented in figure 7.6 where 15% more 30-40k mol wt forms and 17% less large mol wt forms (>40k) were present in the DS placenta than in controls. These differences were not as apparent with pro-αC forms (figure 7.6).

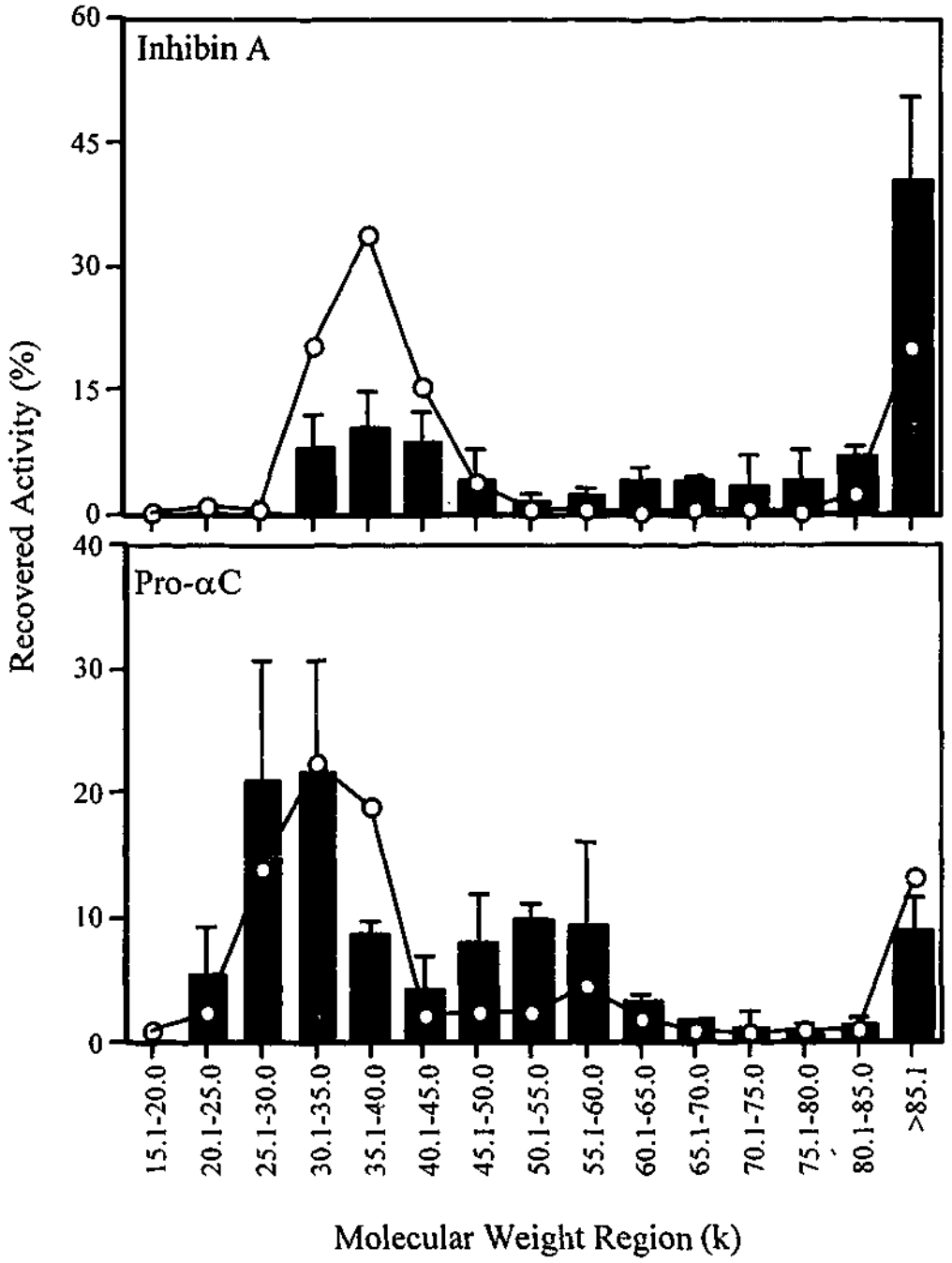



**Figure 7.6:** Mean ± SD molecular weight distribution of inhibin A and pro-αC immunoactivities in placental extracts of control (closed bars) and DS (open circles) samples processed through the combined IA/prep-PAGE procedure. The profile is divided up into mol wt ranges for averaging purposes.

### 7.4.5 Matched Serum and Amniotic Fluid Profiles

The molecular weight forms of inhibin A and pro-αC obtained in MS were compared with matched AF samples. Inhibin B was undetectable in MS but was present in abundance in AF as the mature 36k form (figure 7.7). Both MS and AF showed primarily the mature 36k inhibin A and 25-36k pro-αC forms (figure 7.7).

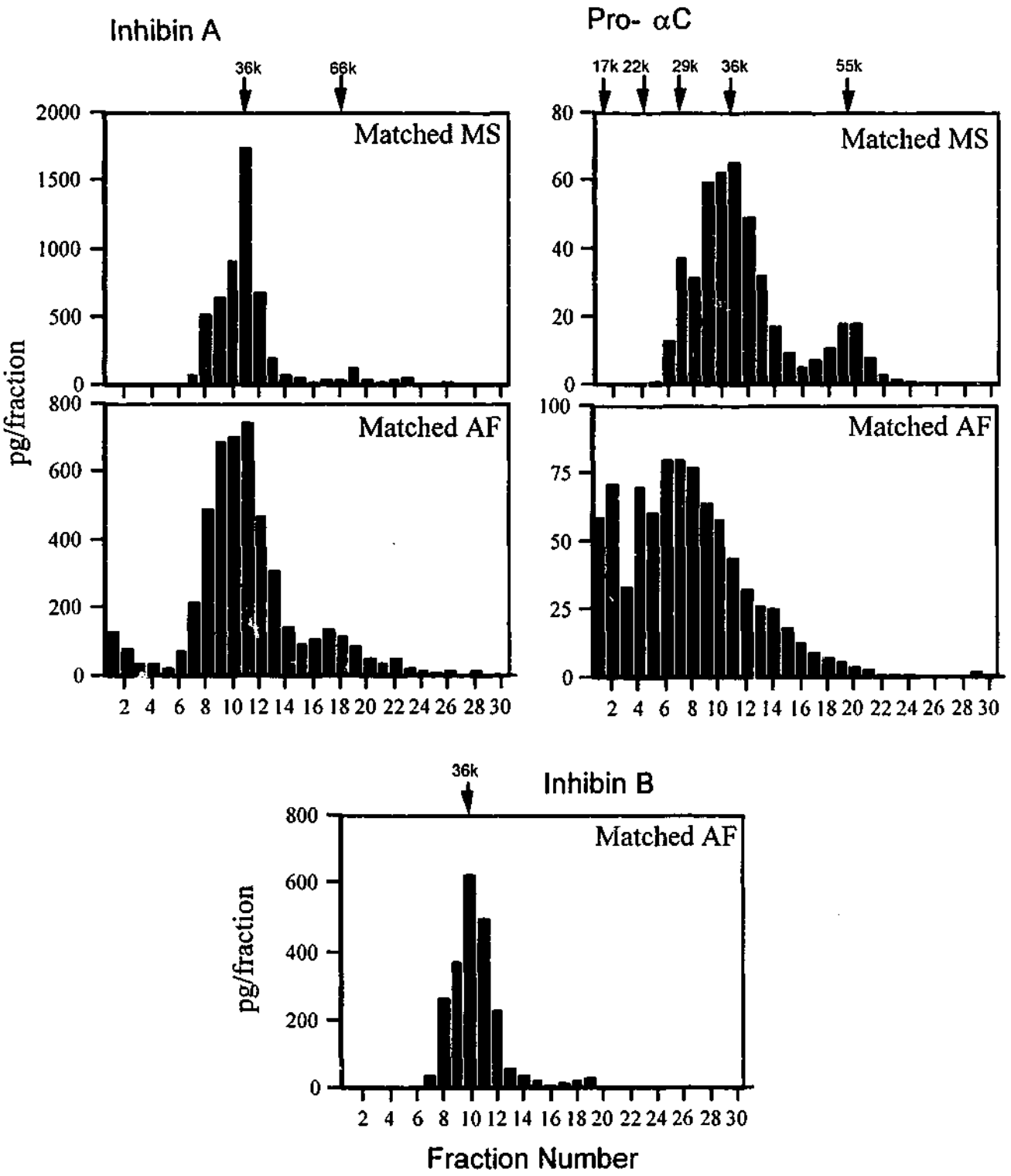



**Figure 7.7:** Molecular weight distribution of inhibin A, inhibin B and pro-αC immunoactivities in matched MS and AF pools fractionated through a combined IA/prep-PAGE procedure. Levels below the sensitivity of the assays were not included.

When specific mol wt regions were compared between AF and MS, AF showed significantly more small mol wt forms (<30k inhibin A and <25k pro-αC) compared to serum (paired *t*-test, inhibin A p=0.035; pro-αC p=0.01) (figure 7.8). There was little difference in the 30-40k inhibin A and 25-40k pro-αC forms or >40k inhibin A and pro-αC forms between MS and AF (figure 7.8; p>0.05 inhibin A and pro-αC).

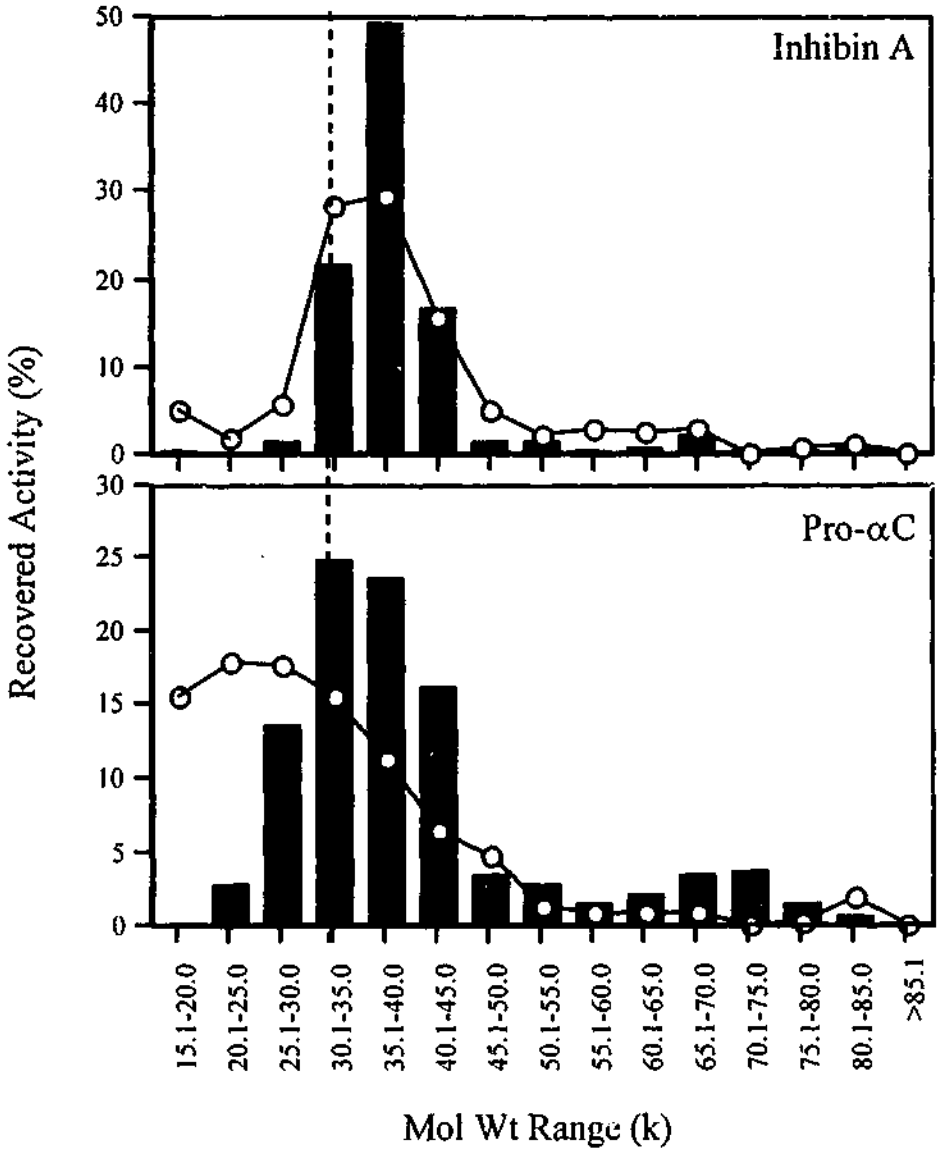



**Figure 7.8:** Molecular weight distribution of inhibin A and pro-αC immunoactivities in matched MS (closed bars) and AF (open circles) samples processed through the combined IA/prep-PAGE procedure. The profile is divided up into mol wt ranges for averaging purposes.

### 7.4.6 Comparison between the different pregnancy compartments

In both normal and DS AF, there was an abundance of <30k forms of both inhibin A and pro-αC compared with MS or placental extracts. There was little difference in the other mol wt regions between compartments (figure 7.9). Of course, no comparisons could be drawn with inhibin B in the different pregnancy compartments as inhibin B was undetectable in both MS and placental extracts.

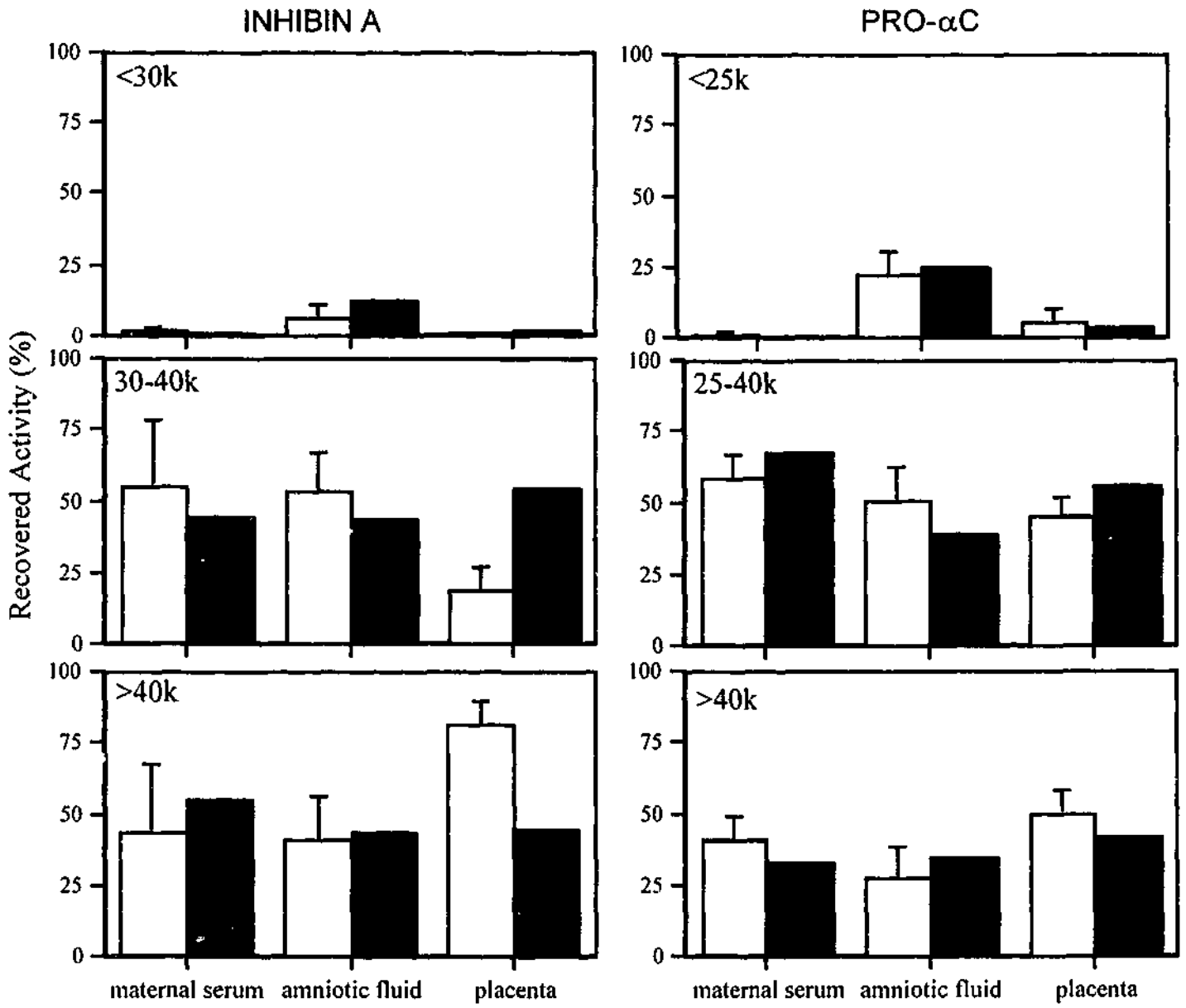



**Figure 7.9:** The proportions of recovered inhibin A and pro-αC in MS, AF and placental extracts of normal (open bars) and DS (closed bars) samples. The pools were fractionated through the combined IA/prep-PAGE procedure.

## 7.5 DISCUSSION

A number of investigators have shown that maternal serum inhibin A levels are significantly altered in aneuploid pregnancies (Wallace et al., 1996; Aitken et al., 1996; Lambert-Messerlian et al., 1996; Noble et al., 1997). Using the same ELISA (Groome and O'Brien, 1993), all of these studies measured 'total' inhibin A. Investigation of the potential for particular molecular weight forms of inhibin A to be useful prenatal markers of DS has been ignored, probably for two reasons. Firstly, until the studies related in this thesis, there has been limited information about the circulating inhibin A forms in pregnancy and secondly, no suitable panel of antibodies exits to detect specific isoforms. Using an improved immunoaffinity fractionation method, this study was undertaken to investigate the possibility that improved discrimination between controls and DS cases might be achieved through the measurement of specific mol wt forms of inhibin A.

Results from the fractionation of 2$^{nd}$ trimester maternal serum from control and DS pregnancies showed that inhibin A and pro-αC immunoactivity was restricted to the 20-40k and 50-70k region for both groups, consistent with the forms published for human plasma and IVF serum (Robertson et al., 1997b) and the pregnancy serum forms detailed in Chapter Four. The peak forms of 36k and 66k most likely correspond to structures of α-$\beta_A$ and pro-αN-αC/$\beta_A$, respectively. There were no obvious differences in the abundancies of certain mol wt forms between normal and DS pregnancy, contrary to the prior hypothesis that specific mol wt forms of inhibin A might be more affected in DS cases. Therefore, the potential for more specific inhibin A assays is not likely.

Fractionation of placental tissue however revealed significant differences between DS cases and control. These differences are unlikely to be the result of differential losses during the fractionation procedure as similar recoveries were obtained from both normal and abnormal groups. The DS placenta contained more mature inhibin A than controls, with immunoactivity predominantly in the 30-40k region, and little evidence of higher mol wt or unprocessed inhibin forms. In control placentae, the pattern appeared more complex with an abundance of both higher and lower mol wt forms (as related in Chapter Five). The DS placental profile was unlike the profiles obtained for

1st, 2nd or term control placenta. These differences in inhibin A mol wt forms between DS and control placental tissues are not as evident for pro-αC inhibin mol wt forms, suggesting specific and abnormal production, processing or cleavage of the forms with the $\beta_A$ fragments only in the DS placenta. This would support the suggestion that the elevated serum inhibin A levels in Down syndrome (Wallace et al., 1996; Aitken et al., 1996; Lambert-Messerlian et al., 1996; Noble et al., 1997), is principally secondary to altered $\beta_A$ subunit secretion by the placenta. In support of altered $\beta_A$ subunit production in DS, a recent study by Dalgliesh et al (2001) revealed stronger staining of the $\beta_A$ subunit than the α-subunit in the placental trophoblasts and stroma of DS tissues. It is therefore likely that the differences observed with placental DS inhibin A mol wt forms is the result of altered post-translational processing of the proβ-$\beta_A$ precursor form to mature inhibin A via an unknown process.

The source of these different inhibin molecular weight forms is most likely the syncytiotrophoblast. Placental extracts (Healy et al., 1988) and primary trophoblast cell cultures (Petraglia et al., 1987) yield large concentrations of inhibin and the mRNAs for the inhibin subunits and the proteins have been localized to the syncytio- and cytotrophoblast (Petraglia et al, 1987, 1991; Minami et al., 1992).

As discussed previously, it is understood that the placenta contributes to the majority of inhibin detectable in MS (Riley et al., 1996; Wallace et al., 1997a; Dalgliesh et al., 2001) and so the observation that the differences in inhibin A mol wt forms between DS cases and controls in placental extracts was not reflected in maternal serum is particularly surprising. The lack of large molecular weight forms in control serum, present in control placental tissue, suggests either that additional cleavage of these large forms occurs in the maternal circulation or that the placenta preferentially secretes smaller mol wt forms into the maternal circulation. While the studies described here cannot address this, the former observation is more plausible. There are numerous other examples of post-secretory cleavage of placental proteins, eg IGF binding proteins 4 and 5 which are cleaved by a serum protease (Byun et al., 2000), the degradation of hCG by a βhCG subunit nicking enzyme (Kardana and Cole, 1994) or the processing of pro-CRH at the syncytiotrophoblast surface by endoproteolytic enzymes (Ahmed et al., 2000). However, if this was the case for inhibin, which enzymes were responsible and whether they are released from the placenta or are

present in the circulation remains unknown. It is certainly possible that processing may occur through endoproteolytic enzymes released from the placental cell surface in an active form (Castro et al., 1989).

Similar to MS, no differences were observed in AF inhibin A, inhibin B and pro-αC mol wt forms between normal and aneuploid pregnancy. Indeed, there were no differences between the three aneuploidies (trisomy 13, trisomy 18 and DS) with all three groups demonstrating the 36k and 66k inhibin A mol wt forms, the 36k inhibin B form and the 27-36k pro-αC form. Consistent with findings from previous studies, inhibin A and pro-αC levels, but not inhibin B levels, in aneuploid amniotic fluid were significantly decreased compared with controls (Wallace et al., 1997b, 1998a, 1999).

Levels of inhibin in amniotic fluid were also higher than in maternal serum, consistent with other existing reports of immunoreactive and dimeric inhibin levels in these compartments (Yokhaichiya et al., 1991; Wallace et al., 1997a). Interestingly, small molecular weight forms of inhibins A and B (<30k) and pro-αC (<25k) were significantly higher in AF than in MS or placental extracts, suggesting independent sources and/or alternative processing of inhibin mol wt forms in the different pregnancy compartments. Indeed, it is clear that while the placenta is a major source of circulating inhibins during pregnancy (Qu and Thomas, 1995; Illingworth et al., 1996a; Muttukrishna et al., 1997; Riley et al., 2000), the fetal membranes also secrete inhibins. The chorion trophoblast, in particular, secretes significant amounts of both inhibin A and inhibin B into amniotic fluid (Riley et al., 1996; Wallace et al., 1997a) and this secretion appears to be independent of that from the placenta (Wallace et al., 1997a; Riley et al., 2000). Taken together, this suggests that the predominant source of the small mol wt forms of inhibins A, B and pro-αC in amniotic fluid is most likely the trophoblast cells of the chorion layer of the fetal membranes, similar to hCG where levels of free βhCG and free αhCG subunits are much higher in AF than in MS while intact hCG levels are lower (Ozturk et al., 1988). The increased concentration of free hCG subunits in AF compared to MS was thought to be due to the chorionic trophoblast preferentially secreting free subunits into AF and the placental trophoblast secreting intact hCG into MS (Ozturk et al., 1988). This is most likely the case with inhibin A and pro-αC.

Other main sources of amniotic fluid production are via fetal urine or the fetal lung (Gilbert and Brace, 1993). However, these are unlikely to be major contributors of the inhibin A or pro-αC small mol wt forms as it has been demonstrated that there is no inhibin A in cord serum (both artery and vein) (Wallace et al., 1997a, 2000). A possible source of inhibin B molecular weight forms in amniotic fluid may be the fetus, with the testis as the likely source (Wallace et al., 1997a; Andersson et al., 1998).

In conclusion, these data suggest while the DS placenta may produce a different profile of inhibin A isoforms than the normal placenta, this difference is not reflected in the maternal circulation. Accordingly, particular inhibin A mol wt forms, rather than 'total inhibin A', are not likely to prove more useful as prenatal markers of DS. The data reported here offer a comprehensive report of the mol wt forms of inhibins A, B and pro-αC in mid-pregnancy, providing evidence to challenge the concept that the mature 32k inhibin is the only circulating form in pregnancy.

# CHAPTER EIGHT

## Ethnic Variation in Maternal Serum Inhibin A

## 8.1 SUMMARY

Prenatal screening for trisomy 21 has become an established part of obstetric practice. Currently most screening programs are conducted at 15-18 weeks' gestation using maternal age in combination with AFP, fβhCG and, more recently, inhibin A. It is known that the concentrations of these markers are affected by maternal weight and, in some instances, by ethnicity. At the Monash Medical Centre, South-East (SE) Asian women form a major component (30%) of the obstetric population and so levels of these three markers were examined in five ethnic groups (Caucasians, Indians, Vietnamese, Cambodians and Chinese) before and after weight correction. Correction for maternal weight involved dividing the observed multiple of the median value (MoM) by the MoM value expected for maternal weight, derived from Caucasian women using the reciprocal correction procedure. Weight-adjusted medians between ethnic groups were then compared using the Mann-Whitney U-test. The results show that, before weight correction, the median MoMs for AFP, fβhCG and inhibin A were significantly higher (by 13-22%, 18-25% and 19-22%, respectively) in the SE Asian groups than in Caucasian women. In Indian women, fβhCG was lower by 8%. Weight correction brought the median marker MoMs of inhibin A and AFP closer to those of Caucasian women, with no significant differences between Caucasians and SE Asian women. However, the difference in fβhCG between Caucasians and Indians (19% lower median MoM) and Caucasians and Chinese (9% higher median MoM) remained significant ($p=0.0001$ and $p=0.0012$, respectively). It is concluded that correction for weight is a most important adjustment. Although ethnic differences in inhibin A and βhCG levels are still present, even after weight correction, the magnitude of this difference in inhibin A levels between SE Asian groups is less than that for βhCG, making inhibin A a particularly useful tool in ethnically diverse populations.

## 8.2 INTRODUCTION

Prenatal screening for trisomy 21 has become an established part of obstetric practice. As detailed in Chapter One (section 1.8.2), despite developments in ultrasound screening, with a few centres adopting measurement of nuchal translucency as part of their routine care (RCOG 1996; Thilaganathan et al., 1998) and the trend becoming increasingly more popular (Mulvey et al., 2000), second trimester biochemical screening still remains unquestionably the most widely used approach (RCOG 1993; Pandya et al., 1995; Thirunavukarasu and Wallace, 1998). While there is a trend to first trimester screening, most serum screening programmes are conducted at 15-18 weeks' gestation using maternal age in combination with the measurement of alpha-fetoprotein (AFP), the free β subunit of hCG (free β-hCG) and either unconjugated estriol ($uE_3$) or, more recently, inhibin A (Cuckle et al, 1996; Wald et al, 1996b). The inclusion of inhibin A in the 'triple test' has significantly improved the specificity of Down syndrome (DS) detection, increasing detection rates by up to 22% for a fixed 5% FPR (Aitken et al., 1996; Wald et al., 1996b; Cuckle et al., 1996). More recently, Debieve and colleagues (2000) reported that the classic triple test was more significantly improved by replacing $uE_3$ with inhibin A than by including ultrasound, further demonstrating that inhibin A would be a powerful marker in this setting. Clearly, the use of inhibin A in the multiple-marker screening test is likely to become the future of standard care and therefore, efforts to improve its performance is strongly beneficial.

Attempts to improve the performance of inhibin A in DS screening has not only been restricted to the investigation of the potential for particular mol wt forms of inhibin A to be more discriminatory markers of DS pregnancy (Chapter Seven), but also to improving the inhibin A assay (Chapter Two), and, in this study, to address, and make adjustments, for any variables in the population of pregnant women to be screened. Indeed, several factors affect serum marker concentrations. These factors include maternal weight, the presence of insulin dependent diabetes mellitus, multiple pregnancy, ethnic origin, parity and smoking. It has been suggested that some of these factors be allowed for as these differences within a screened population may contribute to the variance of the marker levels, reducing the performance of screening

by increasing the overlap in the distributions of the markers in affected and unaffected pregnancies. These factors are briefly outlined below.

### 8.2.1 Maternal Weight

Serum AFP, $uE_3$ and hCG concentrations change with increasing maternal weight (Macri et al., 1986; Palomaki et al., 1990; Reynolds et al., 1991; Wald et al., 1992b). It is well known that light women tend to have higher marker levels and heavier women lower levels, with the reason accepted to be that the distribution volume of serum markers is proportional to maternal body water volume and this volume is greater in heavy women. Indeed, it has been calculated that, on average, for a 20kg increase in weight, serum AFP decreases by 17%, $uE_3$ decreases by 7% and hCG by 16% (reviewed by Wald et al., 1997). Several studies have concluded that although the effect of adjusting for maternal weight is small on Down syndrome screening performance (Wald et al., 1992b), it is worthwhile as the adjustment is simple to perform and has been detailed by Neveux and colleagues (1996). In general, a reciprocal-linear equation, derived from regressing mean maternal weight and median MoM, is used as it is a more appropriate representation of the maternal weight effect (Reynolds et al., 1992; Neveaux et al., 1996).

### 8.2.2 Ethnicity

Ethnic minorities form a significant proportion of the obstetric population of some health districts. AFP and total hCG are greatly affected by ethnicity (Bogart et al., 1991; Gilbert et al., 1996). Although the effect on Down syndrome screening performance is small, with the detection rate increasing by only 0.5% for a false positive rate of 5%, the adjustment is worthwhile because of its established value with screening for open neural tube defects using AFP levels where the false positive rate in black women is about 2.5 times that in white women for a fixed AFP cut-off level after maternal weight adjustment (Wald and Cuckle, 1987). Median hCG levels are higher in blacks (Simpson et al., 1990; Kulch et al., 1993) and Asians (Bogart et al., 1991) than in whites.

### 8.2.3 Gestational Age

The effect of gestation is greatest for markers whose concentrations change most notably with gestational age (notably $uE_3$) and smallest for hCG and inhibin A which change least during the critical period from 15-18 weeks gestation, when second trimester screening is carried out (reviewed by Cuckle, 2000). Interpretation of hCG and inhibin A levels are less affected than that of $uE_3$ by inaccuracies in gestational dating (Wald et al., 1997; Cuckle, 2000).

### 8.2.4 Diabetes Mellitus

AFP, $uE_3$, α-hCG and inhibin A levels are all 9-11% lower in women with insulin dependent diabetes (Wald et al., 1996b; Wallace et al., 1997; reviewed by Wald et al., 1997). The differences are statistically significant and therefore adjustments should be made.

### 8.2.5 Smoking

Serum marker levels tend to be different in women who smoke than in women who do not. The greatest difference is for total hCG where on average, smokers have about 18% lower levels (Cuckle et al., 1990; Bartels et al., 1993). Effect on other markers are small. However, this is compounded by the finding by Cuckle and colleagues (1990) that the birth prevalence of Down syndrome is 16% lower in smoking women, thought to be due to a higher miscarriage rate in smoking women. Because of this, and the small effect of smoking on screening performance, adjustment for smoking status at this stage is unwarranted.

### 8.2.6 Parity

Only total hCG is negatively associated with parity, decreasing by 3% for each previous birth (Haddow et al., 1995; Barkai et al., 1996a; Wald and Watt, 1996). However, the effect on screening performance of adjusting hCG levels is negligible and therefore not worthwhile adjusting MoM values for the number of previous pregnancies.

### 8.2.7 IVF

There is a suggestion that serum marker levels are not affected by assisted reproduction using *in vitro* fertilization (Barkai et al., 1996b; Muller et al., 1993). Levels of both AFP and hCG in this group do no differ from normal pregnancies (Muller et al., 1993), however $uE_3$ levels were found to be lower (Barkai et al., 1996b). Currently, the effect of marker levels is insufficient to warrant adjustment in risk estimation.

The effect of parity and smoking are too small to justify making adjustments for them in interpreting a screening test. However, gestational age, maternal weight and ethnicity affect the serum markers most notably. It has been established that maternal weight and ethnic group can influence the maternal serum concentrations of AFP and free βhCG (Shapiro et al., 1975; Canick et al, 1990, Simpson et al., 1990; Watt et al., 1996). Part of the difference in marker levels between different ethnic groups has been shown to be due to maternal weight, with light women tending to have higher marker levels and heavier women lower levels (Reynolds et al., 1991; Wald et al., 1992b; Bartels et al., 1993). The reason for this is accepted to be that the distribution volume of a marker is proportional to maternal body water volume and therefore in heavy women, this volume is greater. Maternal serum AFP levels are higher in African-Americans than in whites by 30-45% (Crandall et al., 1983; Johnson, 1985; Baumgarten, 1986), while βhCG levels between these two groups differ by 10-25% (Simpson et al., 1990; Kulch et al., 1993). Likewise, levels of βhCG and AFP are elevated in Oriental women compared with whites or Hispanics (Bogart et al., 1991; O'Brien et al, 1997).

Whether inhibin A is similarly affected by weight and ethnicity, and if so, to what extent that has on the performance of the screening programme, is not clear. Only one study to date has studied the effect of ethnicity on inhibin A levels and this paper only addressed one group of Asian women (defined as women originating from the Indian sub-continent) (Watt et al., 1996). Indeed, other studies on the effect of ethnicity on various screening analytes (AFP and hCG) have also principally looked at this particular group of Asian women (Shapiro et al., 1975; Cuckle et al., 1987), defining

'Asian' women are those originating from India. There are no data to date on whether other Asian ethnic groups differ from Indians and/or Caucasians. In this chapter, 'Asian' women are more clearly defined into 4 distinct groups according to the country of origin: Cambodian, Vietnamese, Chinese and Indian. Chinese, Indian and Vietnamese women form a significant proportion (30%) of the multi-ethnic obstetric population at Monash Medical Centre and if there was significant variation in marker levels in these groups, then an important impact on screening efficacy may be present.

The studies in this chapter detail the effect of ethnicity on biochemical screening in general (inhibin A, AFP and βhCG) in the second trimester, before and after adjustment for maternal weight, but of course were designed principally to address any variation in inhibin A.

## 8.3 METHODS

All pregnant women attending Monash Medical Centre for their care are offered screening for chromosomal abnormalities by a combination of fetal nuchal translucency thickness and maternal serum free inhibin A, AFP and β-hCG. A search was made of the database to identify all singleton pregnancies between 1998-2001 in which second trimester biochemical testing had been performed and for which ethnic group and maternal weight information was also recorded. Information on ethnic group was classified for analysis into five distinct groups: Caucasian, Cambodian, Vietnamese, Chinese and Indian. The study population comprised of 2360 Caucasians, 351 Vietnamese, 96 Cambodians, 167 Chinese and 160 Indians. Other subgroups were included within the Caucasian and Indian group because of their small numbers (see Table 8.1).

Inhibin A was measured by the Screening Laboratory at the Victorian Clinical Genetics Service (VCGS), The University of Melbourne (Parkville, Victoria), using a commercial two-site enzyme immunoassay (Serotec), with a similar protocol as detailed in Chapter Two (format I, section 2.2.2). The AFP and free βhCG levels were assayed by the same laboratory at the VCGS using dual time-resolved

fluoroimmunometric assays (DELFIA hAFP/free βhCG Dual kit, Code: A067-101, Wallace Oy, Turku, Finland).

The concentrations of the analytes change with advancing gestation (inhibin A and AFP levels increase while βhCG levels decrease), and so values have to be corrected for gestation prior to deriving likelihood ratios for a normal or DS pregnancy. This is achieved by converting all analyte measurements to multiples of the normal median value (MoM) for each gestation in weeks. Several methods have been proposed for derivation of the medians used to calculate these MoMs (reviewed by Reynolds et al., 1994) but the most acceptable method, with derivations that generate satisfactory MoM distribution, involves regression of gestational age (GA), in weeks, against log-transformed patient results (Reynolds et al., 1992). The gestation-specific medians obtained through this regression analysis of GA, in weeks, with $\log_{10}$ marker levels for β-hCG, AFP and inhibin A are presented below. A quadratic equation was found to fit best.

Inhibin A median (pg/mL) = 10^(3.753 - (0.02673 x GA) + (0.0001101 x GA^2))
AFP median (IU/mL) = 10^(3.593 - (0.02474 x GA) + (0.00002589 x GA^2))
β-hCG median (IU/mL) = 10^(0.1309 + (0.01119 x GA) + (0.000004427 x GA^2))
(^ = power)

Actual analyte concentrations are then divided by the calculated gestation-specific medians to obtain MoM values. MoM values were calculated with and without correction for maternal weight. Correction for maternal weight involved dividing the observed MoM value by the MoM value expected for maternal weight. The expected MoM values were derived solely from Caucasian women using the reciprocal correction procedure (Neveux et al., 1996). This reciprocal transformation of maternal weight more accurately accounts for the effect of maternal weight as it is a more appropriate representation of dilution (Neveaux et al., 1996), which is the accepted explanation for the maternal weight effect. In order to perform this regression analysis, maternal weight was stratified into 14 categories of 5kg lots, ranging from 40-109kg. Regression formulae derived from the reciprocal of the mean maternal

weight (wt) in the 14 categories vs the median marker MoMs for βhCG, AFP and inhibin A are presented below along with the correlation coefficients:

Inhibin A median (pg/mL) = 35.93 x (1/wt) + 0.4528 ($r^2$ = 0.9552)

AFP median (IU/mL) = 38.02 x (1/wt) + 0.4211 ($r^2$ = 0.9245)

Free β-hCG median (IU/mL) = 63.03 x (1/wt) + 0.08325 ($r^2$ = 0.7774)

Weight adjusted medians in the four Asian groups were compared to the Caucasian group using the Mann-Whitney U-test (GraphPad Prism, GraphPad Software, CA). Comparisons of weight-adjusted medians were also made between the Asian groups and analysed using the same statistical test.

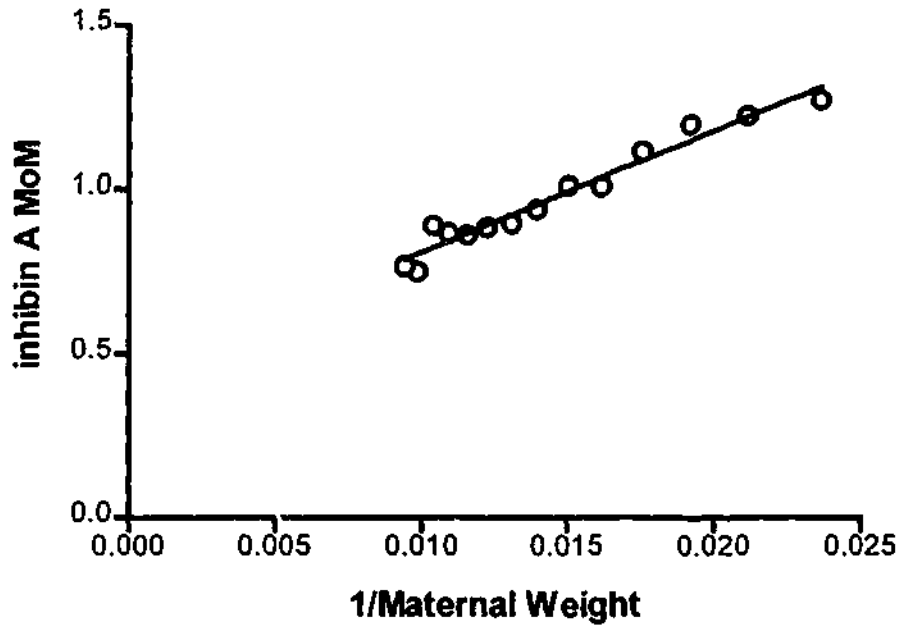
## 8.4 RESULTS

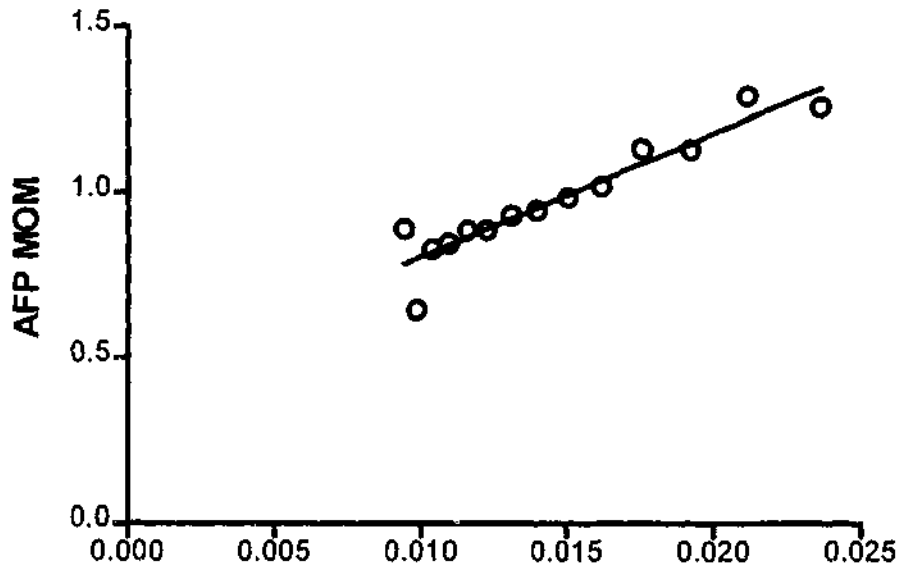
Table 8.1 below summarises the numbers and weights (with 25$^{th}$-75$^{th}$ centiles) of each ethnic group. The maternal weight differed between the ethnic groups with the median weight of the Caucasian women being significantly heavier than that of the SE Asian groups (P=0.0001).

**Table 8.1:** Numbers of each ethnic group with percentages of any subgroups and median weights (kg).

| Ethnic Group | N | Median Weight ($25^{th}$-$75^{th}$ centiles) (kg) |
|---|---|---|
| **Caucasians** | **2360** | 65.0 (58.0 -74.0) |
| • White Australians | 1752 (74.2%) | |
| • New Zealanders | 236 (10%) | |
| • North Americans | 5 (0.2%) | |
| • Europeans | 347 (14.7%) | |
| • Turkish | 21 (0.9%) | |
| **Chinese** | **167** | 55.0 (50.0 - 60.0) |
| **Cambodians** | **96** | 52.0 (48.0 - 55.0) |
| **Vietnamese** | **351** | 50.0 (46.0 - 55.0) |
| **Indians** | **160** | 55.5 (50.0 - 63.0) |
| • Sri Lankans | 65 (40.6%) | |

In the Caucasian group, the concentrations of all three serum markers (AFP, β-hCG and inhibin A) increased with the reciprocal of maternal weight (Figure 8.1). The slope of the increase in marker MoMs with reciprocal maternal weight was similar for all ethnic groups.



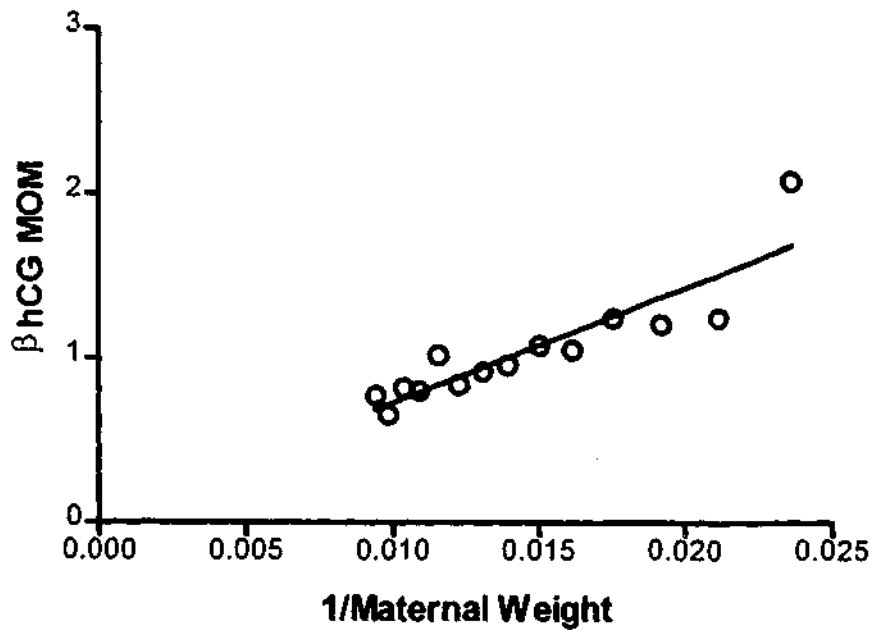




**Figure 8.1:** Regression of the reciprocal of maternal weight and median markers in 2360 Caucasian women.

Without weight adjustment, levels of all three analytes were significantly higher in the Cambodian, Chinese and Vietnamese women than in Caucasian women. The levels of inhibin A, AFP and βhCG were 19-23% higher, 13-22% higher and 18-39% higher, respectively (Table 8.2). In Indian women, only AFP levels were significantly higher (by 14%) than that in Caucasians. No differences were observed for inhibin A or βhCG levels between Indians and Caucasians (Table 8.2).

**Table 8.2:** Marker values (expressed as MoM) in Caucasian, Chinese, Cambodian, Vietnamese and Indian women without maternal weight adjustment. P=significance.

| | Number of: | | Ratio of median MoM (95% CI) Cambodian/ Caucasian | |
|---|---|---|---|---|
| **Serum Marker** | **Caucasian** | **Cambodian** | **Non-weight adjusted** | **P** |
| inhibin A | 2359 | 91 | 1.19 (1.17-1.80) | <0.0001 |
| AFP | 2361 | 96 | 1.22 (1.13-1.29) | <0.0001 |
| βhCG | 2359 | 96 | 1.18 (1.17-1.58) | 0.001 |

| | Number of: | | Chinese/ Caucasian | |
|---|---|---|---|---|
| **Serum Marker** | **Caucasian** | **Chinese** | **Non-weight adjusted** | **P** |
| inhibin A | 2359 | 156 | 1.22 (1.16-1.34) | <0.0001 |
| AFP | 2361 | 167 | 1.13 (1.06-1.18) | <0.0001 |
| βhCG | 2359 | 167 | 1.39 (1.38-1.71) | <0.0001 |

| | Number of: | | Vietnamese/ Caucasian | |
|---|---|---|---|---|
| **Serum Marker** | **Caucasian** | **Vietnamese** | **Non-weight adjusted** | **P** |
| inhibin A | 2359 | 338 | 1.23 (1.15-1.28) | <0.0001 |
| AFP | 2361 | 351 | 1.18 (1.16-1.24) | 0.0002 |
| βhCG | 2359 | 351 | 1.25 (1.22-1.50) | <0.0001 |

| | Number of: | | Indian/ Caucasian | |
|---|---|---|---|---|
| **Serum Marker** | **Caucasian** | **Indian** | **Non-weight adjusted** | **P** |
| inhibin A | 2359 | 151 | 1.00 (0.96-1.17) | 0.75 |
| AFP | 2361 | 160 | 1.14 (1.07-1.20) | <0.0001 |
| βhCG | 2359 | 160 | 0.92 (0.89-1.09) | 0.369 |

Weight adjustment materially altered the Caucasian-Asian difference as the difference in weight distributions between these two groups were large (Table 8.1). Weight adjustment tended to reduce the marker levels of the Asian groups in comparison with Caucasian women, bringing the median marker levels of the analytes closer to those in Caucasians (Table 8.3).

**Table 8.3:** Marker values (expressed as MoM) in Caucasian, Chinese, Cambodian, Vietnamese and Indian women after maternal weight adjustment. P=significance.

| | | | Ratio of median MoM (95% CI) | |
|---|---|---|---|---|
| | Number of: | | Cambodian/ Caucasian | |
| Serum Marker | Caucasian | Cambodian | Weight adjusted | P |
| inhibin A | 2359 | 91 | 1.22 (1.16-1.34) | 0.16 |
| AFP | 2361 | 96 | 1.05 (1.01-1.13) | 0.06 |
| βhCG | 2359 | 96 | 1.15 (0.93-1.23) | 0.40 |

| | Number of: | | Chinese /Caucasian | |
|---|---|---|---|---|
| Serum Marker | Caucasian | Chinese | Weight adjusted | P |
| inhibin A | 2359 | 156 | 1.13 (1.04-1.20) | 0.11 |
| AFP | 2361 | 167 | 0.98 (0.95-1.05) | 0.93 |
| βhCG | 2359 | 167 | 1.09 (1.07-1.31) | 0.001 |

| | Number of: | | Vietnamese/ Caucasian | |
|---|---|---|---|---|
| Serum Marker | Caucasian | Vietnamese | Weight adjusted | P |
| inhibin A | 2359 | 338 | 1.04 (0.98-1.10) | 0.39 |
| AFP | 2361 | 351 | 1.01 (0.99-1.06) | 0.33 |
| βhCG | 2359 | 351 | 0.99 (0.94-1.07) | 0.06 |

| | Number of: | | Indian/ Caucasian | |
|---|---|---|---|---|
| Serum Marker | Caucasian | Indian | Weight adjusted | P |
| inhibin A | 2359 | 151 | 0.92 (0.88-1.08) | 0.09 |
| AFP | 2361 | 160 | 1.04 (0.99-1.10) | 0.26 |
| βhCG | 2359 | 160 | 0.81 (0.71-0.88) | 0.0001 |

Although AFP and inhibin A levels in South East Asian women remained slightly higher than in Caucasians, after weight correction, these differences were not statistically significant (maternal weight corrected $\log_{10}$ MoM in each Asian group compared with the Caucasian maternal weight corrected $\log_{10}$ MoM, Mann-Whitney U-test, Table 8.3). However, the differences between Caucasians and Chinese, and between Caucasians and Indians remained highly significant for βhCG (15% higher; p=0.0012 and 19% lower; p=0.0001, respectively) (Table 8.3).

When the maternal weight corrected $\log_{10}$ MoMs of inhibin A, AFP and βhCG were compared *within* the South East Asian groups using a Mann-Whitney U-test, inhibin A levels were significantly different between Indian women and Cambodians (p=0.035), Vietnamese (p=0.042) and Chinese (p=0.024). AFP did not reach statistical significance between any of the groups (p>0.05). However, βhCG levels were significantly different between Indians and Vietnamese (p<0.01) and Indians and Chinese (p<0.001). Based on these results, the maternal weight corrected $\log_{10}$ MoMs of the individual markers for Cambodian, Vietnamese and Chinese women were combined and compared with Caucasians using a Mann-Whitney U-test. Results showed no significant differences between these groups (inhibin A p = 0.11; AFP p =0.12; βhCG p = 0.25).

Median ratios obtained by comparisons between Indian and Caucasian women from this study were compared with published estimates from three other studies which had classified 'South Asian' women as women originating from the Indian sub-continent. Table 8.4 shows that the ratios from this study are very similar to those obtained by other investigators.

**Table 8.4:** Comparison of the ratio of weight adjusted: non-weight corrected MoM between Indian women and Caucasians in this study with that obtained in three other studies.

| | Ratio of wt adjusted: non-wt adjusted MoM | | |
|---|---|---|---|
| **Serum Marker** | **This Study** | **Watt et al (1996)** | **Cuckle et al (1987)** |
| Inhibin A | 0.92 | 0.93 | - |
| AFP | 0.91 | 0.91 | 0.94 |
| βhCG | 0.88 | 0.92 | - |

## 8.5 DISCUSSION

Since maternal AFP and βhCG serum screening began almost 20 years ago, a number of adjustments, such as those for maternal weight, diabetic status and multiple gestation have been added as accumulated data suggested their need for optimal screening (reviewed by Wald et al., 1998). It is now well documented that ethnicity plays a significant role in marker variation. Indeed, differences in biochemical markers levels has been shown to exist for Afro-Caribbean, South Asian, Oriental and Hispanic women in the second trimester of pregnancy, and these differences, compared to Caucasian women, cannot be explained solely by differences in maternal weight (Watt et al., 1996; Bryne et al., 1997; Bryant-Greenwood et al., 1998). Importantly, variations in race could, in the case of AFP screening for open neural tube defects, yield as much as a 10-fold difference in the true likelihood of a defect (O'Brien et al., 1993). Clearly, adjustments for race/ethnicity for some markers will result in more accurate, and therefore improved, screening. However, there are only very limited data regarding marker adjustments in South-East Asian women, with most reports relating levels in either black Americans or 'Asian' women from the Indian subcontinent. Data on inhibin A levels, in particular in the Asian population, are even more limited, with only one study to date comparing levels between Asian (Indians) and Caucasians (Watt et al., 1996). Lam and Tang (1999) studied the utility of inhibin A as a marker of Down syndrome in Asian women but theirs was a mixed

population of Chinese, Indian and Vietnamese women with no weight-adjustment data.

In this study, significant ethnic differences in second trimester βhCG levels are shown between Caucasians, Chinese and Indians. Differences in inhibin A and βhCG levels are also noted between the groups of Asian women. Importantly, these differences are likely to be real because the differences observed for inhibin A, AFP and βhCG in this study in the Indian women (0.92, 0.91, and 0.88, respectively) closely matches those obtained by Watt and colleagues (1996) (0.93, 0.91 and 0.92, respectively) for 'Asian' women (derived from women in the Indian sub-continent). Cuckle et al (1987) derived similar median AFP MoMs in their South Asian women (Indian) to that derived in this study, further adding support to the differences observed here.

These differences in analyte levels are important as Vietnamese, Chinese and Indian women form a significant proportion of the obstetric population at Monash Medical Centre and no studies have looked at these groups separately. In this study, the marker levels in 5 ethnic groups were examined separately and the results show differences between the different groups. The growing ethnic diversity in multicultural Melbourne and the variation in analyte levels (particularly βhCG) between groups of Asian women strongly supports the principle that there should be separate databases among the various major ethnic groups whenever possible.

In the four different South East Asian groups of women in this study, all three marker levels were significantly higher than in Caucasians, with weight correction accounting for some of the differences. The marker most significantly affected was βhCG, with levels (after weight correction) of 9% and 15% higher in Chinese and Cambodian women, respectively. However, βhCG levels were depressed in Vietnamese and Indian women, suggesting that specific racial adjustment in routine βhCG screening for fetal Down syndrome is necessary. Although the effect of adjusting for ethnic group may not have a significant impact on overall detection rates, with only a maximum of about 0.5% extra detection at a 5% false-positive rate (Watt et al., 1996; Spencer et al., 2000), the impact on an individual's risk is significantly altered. In this

study, the calculated risk of aneuploidy in Indian women would be increased (with their low βhCG levels) whilst the risk in Chinese women (with their high βhCG levels) would decrease. The results obtained in this study suggest that screening centers with large numbers of ethnically-diverse patients may have to consider firstly, adjusting data for weight and secondly, further breakdowns of the data to create race-specific database groups in order to increase screening efficacy. On the basis that there were no significant differences in marker levels between Chinese, Vietnamese and Cambodian women, but large differences in βhCG levels between Indian women and the other Asian groups, a possible strategy would be to combine marker levels for Chinese, Vietnamese and Cambodian women, while creating a separate database for Indians. Currently, there is no ethnic correction in prenatal screening markers in Australia (Richard Ryall, personal communication).

The reason for the ethnic differences in the levels of inhibin A between Indians and Chinese/Vietnamese and βhCG differences between Caucasians and Chinese/Indians and is not known. The placenta is the main source of inhibin A and βhCG in the second trimester of pregnancy (see Chapter One, section 1.7) and thus it appears that the placenta of certain groups of Asian women produces more or less of these proteins than other groups. There is no evidence that Indian women have smaller placentae or Chinese women larger placentae, which might account for the differences in inhibin A and βhCG levels. Whether the increased/decreased amount of inhibin A/βhCG is due to increased/decreased placental size or increased/decreased production cannot be determined from this study. Further studies are needed to explain these differences in marker production between various ethnic groups and to develop robust methods of correcting for ethnic origin.

In summary, these results demonstrate that major differences in analyte levels between ethnic populations are due to maternal weight and therefore weight correction is an important, and necessary, adjustment in the screening programme. Differences in ethnicity are still obvious even after weight correction, especially for βhCG levels, indicating a need for race-specific databases. Given that the magnitude of the difference in inhibin A levels within SE Asian women was smaller than that for

βhCG, this study demonstrates inhibin A to be a particularly useful marker in ethnically diverse populations.

# CHAPTER NINE

## Conclusions and Future Prospects

## 9.1 INTRODUCTION

Several investigators have shown that the placenta and the fetal membranes are the major sources of inhibins in human pregnancy (Qu and Thomas, 1995; Riley et al., 1996; Wallace et al., 1997a). It is now well established that the placenta contributes to the majority of inhibin detectable in maternal serum (Riley et al., 1996; Wallace et al., 1997a) while the fetal membranes are the major source of inhibins in amniotic fluid (Wallace et al., 1997a; Wallace et al., 1999). While much of the regulation of placental inhibins is known, the inhibin mol wt forms that are released have never been investigated. Given that immunoaffinity fractionation by Robertson et al (1996, 1997a) revealed multiple molecular weight forms of inhibins in human follicular fluid, IVF serum, postmenopausal serum and male plasma, with the forms differing in the different fluids, this study was primarily undertaken to establish which molecular weight species of inhibin A, inhibin B and pro-αC are present in pregnancy serum.

The studies related in this thesis are observational in nature. The underlying rationale was of course to improve the utility of inhibin A as a marker of abnormal pregnancies by identifying which molecular sizes of inhibin were particularly deranged in association with Down syndrome (DS) and preeclampsia (PE) in particular. If differences did exist, then the implications for screening would have been significant, and would have encouraged the development of new antibodies against specific epitopes, allowing the detection of specific forms of inhibin A. However, the results obtained in maternal serum and amniotic fluid showed, disappointingly, no differences between DS and control groups. However, placental inhibin forms were abnormally produced or cleaved in association with both Down syndrome and preeclampsia. In summary, the studies related in this thesis have afforded novel and interesting insights into the inhibin forms present during human pregnancy.

## 9.2 INHIBIN FORMS IN NORMAL PREGNANCY

### 9.2.1 Pregnancy Serum

It is now clear from Chapter Four that mature inhibin A is not the only circulating inhibin molecular weight form in maternal serum, contrary to the findings of

Muttukrishna et al (1995). In addition to the 36k dimer, inhibin A mol wt forms of 66k, 32k and an abundance of forms <30k and pro-αC forms of 27k, 32k and forms less than <25k were also present in maternal serum. No inhibin B molecular weight forms were identified in pregnancy serum, confirming previous reports (Wallace et al., 1997a; Riley et al., 2000; Wallace et al., 2000) and refuting others (Florio et al., 1999).

There may be several reasons to explain the discrepancy between the inhibin forms obtained in this study and that obtained by Muttukrishna et al (1995). Firstly, the method used by Muttukrishna and colleagues (1995) – gel filtration chromatography – has limited resolution and may not readily detect relatively low levels of the small inhibin A and pro-αC molecular weight forms. Secondly, their serum was a pool from varying gestations which may have had a 'dilution' effect: more 9-18 weeks' gestational serum and less late pregnancy serum in the pool would have reduced the impact of small molecular weight presence.

The changes in small molecular weight forms associated with increasing gestation, reported in Chapter Four, is entirely novel. The increase in the proportion of inhibin A and pro-αC small molecular weight forms of <30k and <25k, respectively, in late pregnancy serum was speculated to be due to either 1) reduced glycosylation by the placenta, leading to deglycosylated, and therefore reduced, sizes of inhibin α subunit, 2) some undefined clinical difference associated with altered inhibin processing in the cross-sectional patient groups, or 3) cleavage by a late pregnancy blood protease.

### 9.2.2 Placenta

To test the first hypothesis, placental homogenates from first, second and term placentae were processed through the immunoaffinity fractionation procedure and characterized (Chapter Five). The major molecular weight peaks of inhibin A and pro-αC in placental tissue in all three trimesters were found to be 97-55k and 36-29k, representing partially processed or unprocessed dimers and processed or unprocessed free α-subunits, with little evidence of small mol wt forms. When compared with maternal serum profiles, placentae from all three trimesters showed significantly more

large mol wt forms (>40k) with little <30k mol wt forms, even in term tissue. This is in keeping with data published by de Kretser et al (1994) who investigated the mol wt forms present in term placental extracts using chromatographic analysis and similarly showed no evidence of small mol wt forms in the term placentae.

The abundance of <30k forms and little >40k in late pregnancy maternal circulation, together with the opposite finding in term placentae, suggests that either:

a) increased precursor processing of the >40k forms in term placenta occurs at the syncytiotrophoblast surface, with the enzymes involved possibly released from the placenta, and subsequent spillage of cleavage products of >40k forms into maternal circulation, or
b) the >40k placental forms are secreted into the maternal circulation where they subsequently undergo processing by a circulating protease.

These suggestions are of course speculative but are based on other studies which have also demonstrated the presence of increased proteolytic activity in late pregnancy serum (IGFBP3, IGFBP4 and IGF 5 [Claussen et al., 1994; Irwin et al., 2000; Byun et al., 2000], hCG [Kardana and Cole, 1994] or cleavage of precursor forms in serum by placental furins (McLachlan et al., 1986b; Robertson et al., 1987).

The possibility that artifacts in the fractionation procedure may be responsible for the formation of small mol wt forms in pregnancy was essentially excluded by the addition of serine protease inhibitors to the samples and at various stages throughout the fractionation procedure (Chapter Three). That cleavage would have occurred during the initial immunoaffinity step was also unlikely given that hFF, fractionated directly through Prep-PAGE or processed through the immunoaffinity column followed by Prep-PAGE, showed similar mol wt patterns (Chapter Three). The validation studies conducted in Chapter Three showed that the immunoaffinity chromatography procedure used here was a valid way of studying the inhibin mol wt forms in pregnancy.

### 9.2.3 Amniotic Fluid

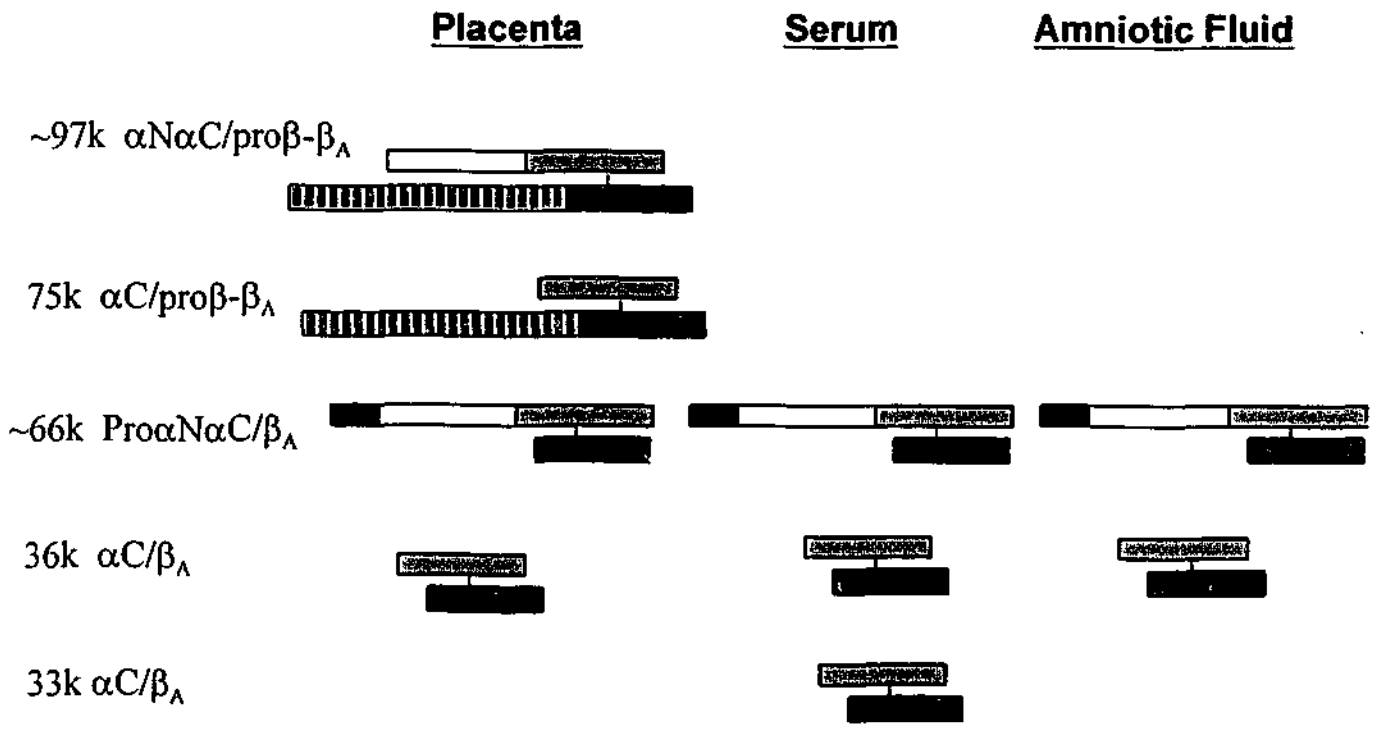
The inhibin sizes present in amniotic fluid have never been explored. The studies related here show that the second trimester amniotic fluid forms of inhibin A and pro-αC detected by the ELISA used in these studies were similar to those identified in matched maternal serum samples (Chapter Seven). Of course, since inhibin B is undetectable in maternal serum at this gestation, no comparisons between amniotic fluid and maternal serum could be made.

The forms detected in AF were the 36k and 66k inhibin A, 36k inhibin B and 29k and 32k pro-αC forms. The proportion of <30k inhibin A and B forms and <25k pro-αC forms in amniotic fluid was greater than that in matched maternal serum samples, suggesting differential sources of these small mol wt forms, consistent with previous suggestions that the chorionic trophoblast secretes inhibin A and inhibin B into amniotic fluid whereas the placenta secretes inhibin A and pro-αC into maternal serum (Riley et al., 1996; Wallace et al., 1997a). Likewise, levels of fβhCG and fαhCG subunits are much higher in amniotic fluid than in maternal serum while intact hCG levels are lower (Ozturk et al., 1988), similarly thought to be due to differential secretion. Taken together, it is speculated that the small molecular weight forms of inhibin A, inhibin B and pro-αC in amniotic fluid are secreted by the chorionic trophoblast cells of the fetal membranes, while a further possible source of inhibin B mol wt forms may be the fetus, with the developing testis as the likely source (Wallace et al., 1997a).

## 9.3 OVERVIEW OF INHIBIN FORMS IN NORMAL PREGNANCY

The inhibin forms present in human pregnancy are presented below in a summary of the forms present in pregnancy (figures 9.1 and 9.2). The inhibin A forms of 97k, 75k, 66k and 36k and 33k most probably refer to structures of αN-αC/proβ-$\beta_A$, αC/proβ-$\beta_A$, Pro-αN-αC/$\beta_A$ and mono or diglycosylated αC/$\beta_A$, respectively. The inhibin B form of 36k found in amniotic fluid refers to monoglycosylated αC/$\beta_B$, while the pro-αC forms of 55k, 33k and 29k refer to Pro-αN-αC and mono or diglycosylated Pro-αC, respectively.

**Normal Pregnancy (Inhibin A)**




**Normal Pregnancy (Inhibin B)**

| | Placenta | Serum | Amniotic Fluid |
|---|---|---|---|
| 36k $\alpha C/\beta_B$ | Not detected | Not detected | |

**Figure 9.1:** Molecular weight forms of inhibin A and inhibin B in the different compartments of pregnancy.

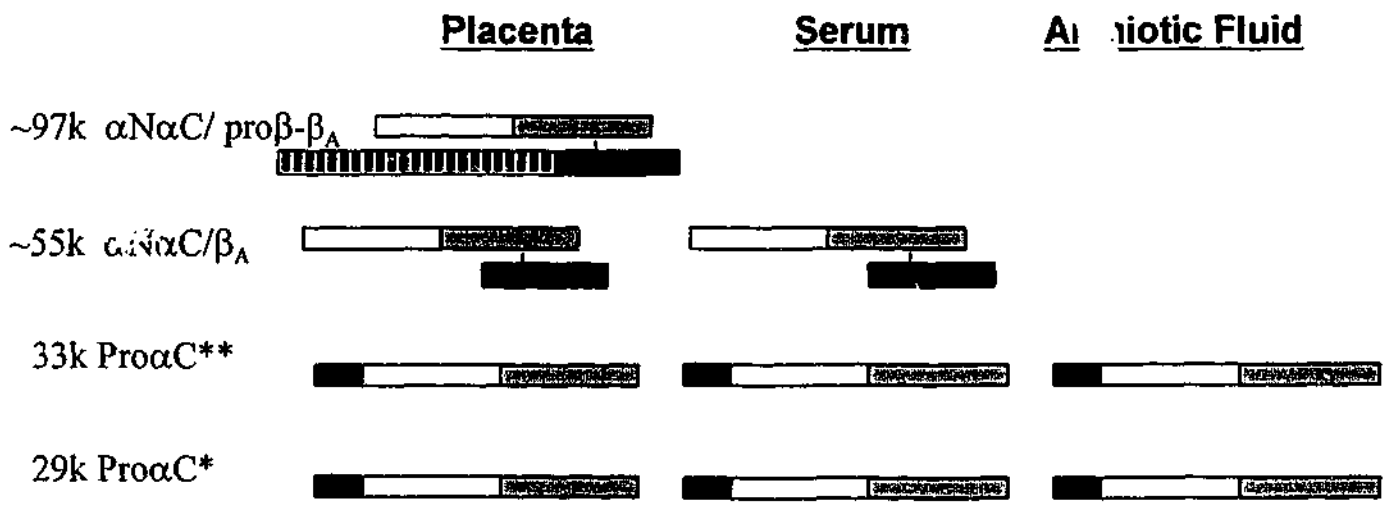



**Figure 9.2:** Molecular weight forms of inhibin pro-αC in the different compartments of pregnancy.

The sources and routes of secretion of the <30k inhibin A and B forms and the <25k pro-αC forms, found in abundance in serum and amniotic fluid, are presented in a schematic diagram below. The source of inhibins in AF is most likely the fetal membranes (Wallace et al., 1997a), with the fetus possibly contributing to the small mol wt forms of inhibin B (via fetal testis, Tuuri et al., 1994).

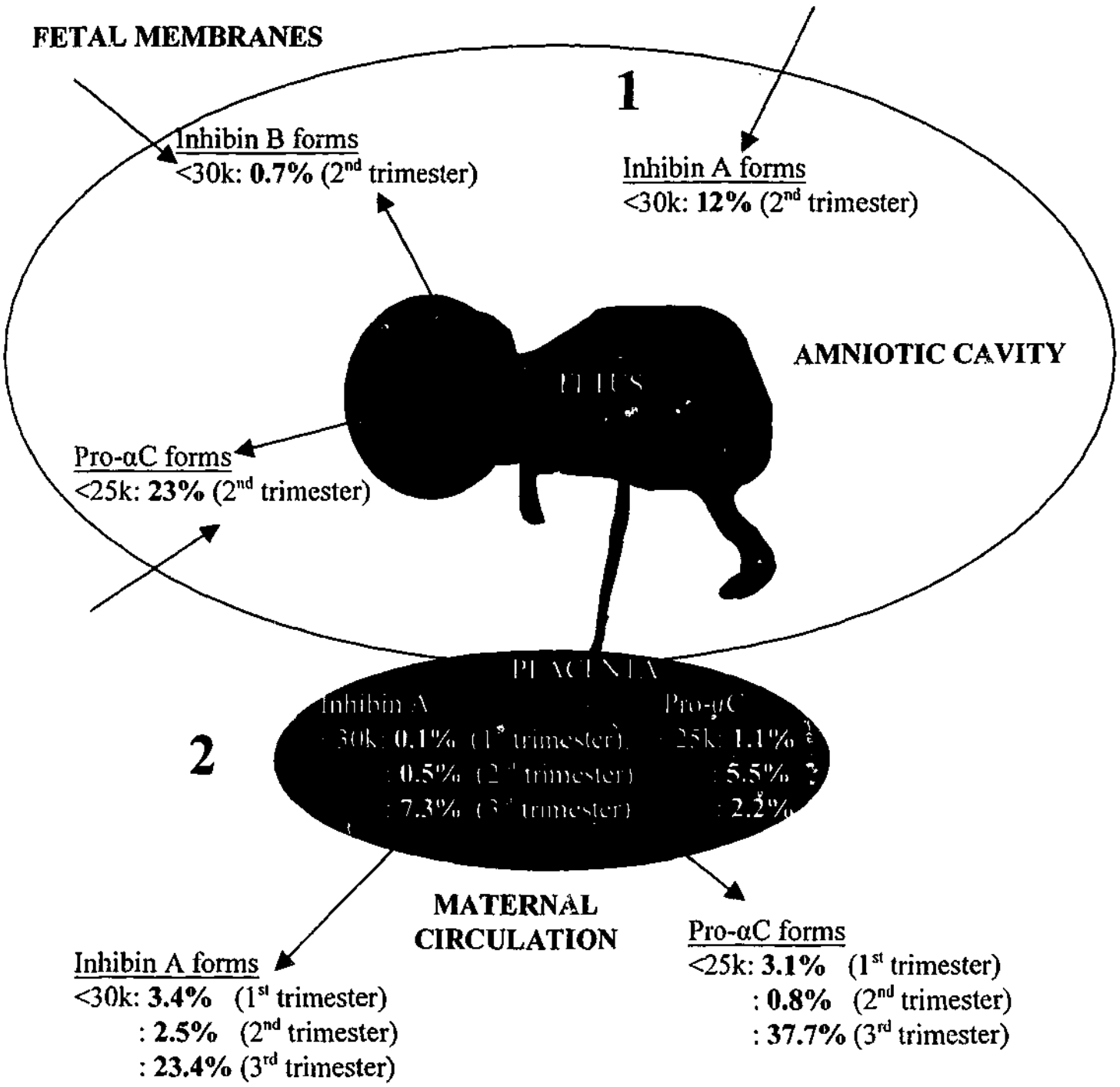



**Figure 9.3:** Schematic representation of the possible sources and routes of secretion of the <30k inhibin A and inhibin B forms and <25k pro-αC molecular weight forms.

**1:** The abundance of these small mol wt forms in amniotic fluid may be due to:

a) cleavage occurring in amniotic fluid itself, which is unlikely or

b) the fetal membranes secrete small mol wt forms into amniotic fluid.

**2:** The abundance of the small mol wt forms in maternal serum may be due to:

a) cleavage of placental forms at the syncytiotrophoblast surface by placental furins and subsequent spillage into the maternal circulation, and/or

b) cleavage by a late pregnancy blood protease.

## 9.4 INHIBINS IN ABNORMAL PREGNANCIES

The descriptive characterization of changes in inhibin isoforms during normal pregnancy was clearly a necessary backdrop to comparative descriptions of the distribution of inhibin isoforms across abnormal pregnancies. Prior to the studies reported in this thesis, the inhibin molecular weight sizes secreted in aneuploid pregnancies had never bee.. explored. The studies related here show that the forms of inhibin A and pro-αC in second trimester DS pregnancies were similar to those identified in gestational age-mat ed normal maternal serum samples (Chapter Seven). These forms were the 36k and 66k inhibin A and 29k and 32k pro-αC forms. Apart from an increase in the proportion of 25-40k inhibin A and pro-αC forms in DS maternal serum, which contain the 36k mature dimer and 29k pro-αC form, there were little differences in the proportions of other forms between the two groups. Similarly, there were no differences in the types of forms of inhibin A, inhibin B and pro-αC in the amniotic fluid of trisomy 13, trisomy 18 and DS pregnancies compared to controls apart from a decrease in 25-40k forms. The results obtained with these molecular wt regions were expected given that previous investigators have demonstrated increased maternal serum inhibin A and pro-αC levels in DS pregnancies but decreased amniotic fluid levels (Aitken et al., 1996; Wallace et al., 1997b; Wallace et al., 1998a). It was disappointing to note that there were no other differences in mol wt forms between DS and control AF and MS samples.

However, while there were no differences in *inhibin species* between amniotic fluid and maternal serum DS and control samples, there were significant differences in the *proportions* of large mol wt forms between DS placental tissue and controls in the second trimester of pregnancy (Chapter Six). In particular, the DS placenta contained little to no large mol wt forms (97k, 75k and 66k inhibin A; 55k pro-αC) with an abundance of 30-40k forms, altogether suggesting abnormal cleavage of forms containing the precursor $\beta_A$ segment in the placental tissues of DS pregnancies, with preferential post-translational processing of the large mol wt forms to the mature 36k inhibin A form.

Similarly, in the preeclamptic placenta, the total products of inhibin A are increased compared to normal, and this increase is associated with relatively less large molecular weight forms, suggesting increased processing. The feature that is common to both DS and PE pregnancies is the presence of more 30-40k forms and less large mol wt forms >40k, suggesting that the placenta in abnormal pregnancies undergoes altered processing. Other researchers have also demonstrated altered protein processing in relation to preeclampsia (placental CRH, Ahmed et al., 2000; Goland et al., 1995). However, the data have to be interpreted with caution as the design of this study was hampered by the fact that the presence of severe PE usually results in preterm delivery, more frequently by caesarean section, and therefore adequate control groups, featuring women delivering preterm, with pregnancies not complicated by maternal or fetal pathologies, were difficult to acquire. This was a major limitation of Chapter Six.

## 9.5 IMPROVING THE UTILITY OF INHIBIN A IN PRENATAL SCREENING

Down syndrome is the single most common cause of severe mental handicap in the developed world. There are now various antenatal screening strategies for Down syndrome available, offering women and couples an opportunity to identify an affected pregnancy early and so consider the most appropriate action. The current optimum combination of second trimester biochemical markers appears to be fβhCG, AFP and inhibin A (Spencer et al., 1992; Aitken et al., 1996). Several factors affect serum marker concentrations (detailed in Chapter Eight, section 8.2) but the most notable factors are gestational age, maternal weight and ethnicity (reviewed by Wald et al., 1997). Whether these factors affect inhibin A to a similar extent, and if so, to what extent that has on the performance of the screening programme was investigated in Chapter Eight.

The results, a first report on AFP, βhCG an inhibin A differences in groups of South East Asian women, show no differences in AFP levels between ethnic groups, only small differences in inhibin A levels and large differences of fβhCG levels between racial groups (Chapter Eight), which argues in favour of inhibin A as a stable marker across ethnically diverse populations.

Results of the three markers were expressed as multiple of the medians (MoMs) to allow comparisons across gestations as the levels of all serum markers change with gestation. However, the effect of gestation is smallest for inhibin A, which changes least during the critical period from 15-18 weeks' gestation when screening is carried out (previously reported by Aitken et al., 1996), and therefore interpretation of inhibin A levels are less affected by inaccuracies in gestational dating. This, combined with the finding of little inhibin A variation between ethnic groups, makes inhibin A the marker with least variance and so most stability and utility.

The utility of measurement of inhibin A in DS screening could also be affected by stability of the protein. The stability studies undertaken in Chapter Two revealed that inhibin A was relatively stable in serum, but if patient specimens were kept as whole blood for prolonged periods at room temperature, the simple addition of 3-amino-1,2,4-triazole, a catalase inhibitor, would prevent the decline of up to 70% in inhibin A values caused by erythrocyte catalase interference (Wallace et al., 1998b; Thirunavukarasu and Wallace, 2001). Essentially, the studies detailed in Chapter Two indicate the need for the addition of an inhibitor like 3-amino-1,2,4-triazole in DS screening programmes in which inhibin A is used as a marker because whole blood specimens may spend varying lengths of time in transit to the screening centre. This addition of 3-amino-1,2,4-triazole to samples or to the plate wells prior to assay is a simple but valuable assay modification (Thirunavukarasu and Wallace, 2001).

## 9.6 FUTURE PROSPECTS

The studies described in this thesis show firstly that various inhibin molecular weight forms are found in pregnancy and these forms differ with increasing gestation. Of these forms, 'total' inhibin A is a better marker of DS and PE pregnancies than specific molecular weight forms. However, it is unclear to what extent the various forms contribute to the *in vivo* activity of inhibin, nor where they are processed (at the site of production and/or in the circulation). It is likely that the small molecular weight forms, found in abundance in amniotic fluid and late pregnancy serum, possess precise biological functions; studies exploring this area would indeed be most useful.

However, the isolation and characterization of the small molecular weight forms will be a difficult process because of their relatively low levels in human pregnancy. Therefore, quantitative studies and the potential physiological role/significance of individual isoforms, while necessary for appreciating the significance of the changes described in this thesis, must await purification of adequate amounts of material.

Interestingly, recent studies by David Robertson (personal communication) from Prince Henry's Institute of Medical Research, Victoria, suggests that the forms of both inhibin A and pro-αC are inadequately detected by current immunoassay methods which are too selective in the forms of inhibin they detect. If this is true, the levels of the low mol wt forms of inhibin reported in this manuscript may be underestimated and the development of new inhibin assays, with specific monoclonal antibodies directed to different regions of the inhibin epitopes, may prove useful as replacement assays in clinical studies.

# APPENDIX

## Sources of Chemicals, Reagents & Instruments

## 1.1 GENERAL AND CHEMICALS REAGENTS

***Hydrogen peroxide ($H_2O_2$), polyoxyethylene(20)sorbitan monolaurate (Tween-20), bovine serum albumin (bSA), 3-amino-1,2,4-triazole (AT)*** and ***bovine liver catalase*** (Type C-10) were purchased from Sigma (Castle Hill, Victoria, Australia). ***Sodium dodecyl sulphate (SDS)*** was obtained from Astral (Gymea, NSW, Australia). ***Fetal calf serum (FCS)*** was purchased from CSL (Parkville, Victoria, Australia). ***ELISA (Enzyme Linked Immunosorbent Assay) amplification systems*** were supplied by Life Technologies (Mulgrave, Victoria, Australia). ***Sodium chloride (NaCl), sodium azide ($NaN_3$)*** and ***hydrogen chloride (HCl)*** were obtained from Ajax Chemicals (Auburn, NSW, Australia). ***Triton X-100*** and ***tris(hydroxymethyl)methylamine (Tris-base)*** were obtained from BDH Chemicals (Kilsyth, Victoria, Australia). ***Normal mouse serum*** was obtained from Australian Laboratory Services (Sydney, NSW, Australia).

## 1.2 GENERAL LABORATORY CONSUMABLES AND SMALL EQUIPMENT ITEMS

***Pipette tips***, ***eppendorfs*** and adjustable ***Finnpipette*** were obtained from Interpath (Heidelberg, Victoria, Australia). ***Plate seals*** were obtained from Pathtech, (Blackburn, Victoria, Australia). Adjustable ***single channel pipettes*** (2-20µl, 10-200µl, 100-1000µl) were obtained from John Morris Scientific (Willoughby, NSW, Australia). A ***multichannel pipette*** (50-200µl), ***automatic vortex mixer*** and ***plate shaker*** were obtained from Selby-Biolab (Mulgrave, Victoria, Australia). ***Multichannel pipette reagent boats*** were obtained from Socorex (UK). A ***plate reader*** was obtained from Beckman Instruments (Gladesville, NSW, Australia) and the associated ***software*** (Softmax) from Molecular Devices (Sunnyvale, California, USA). Water used to prepare or dilute solutions was ***double-deionised RO*** (reverse osmosis) water from a Millipore unit (Millipore Australia) (NSW, Australia).

## 1.3 ITEMS SPECIFIC FOR ELISAS

Inhibin A-, inhibin B- and pro-αC coated ***microtitre plates*** (Nunc, Maxisorb) were purchased from Professor Nigel Groome (Oxford Brookes University, UK) until 1999 and thence from Oxford Bio-Innovation (Upper Heyford, Oxford Shire, UK). These

plates were stored at 4°C as recommended by the supplier. As well, ***lyophilised inhibin A, B and pro-αC standards*** and ***mouse monoclonal antibody*** raised against human α-subunit (2nd antibody) were purchased from the same suppliers and stored at 4°C. The ***quality control*** (QC) were pools of high and low values prepared from human amniotic fluid collected within the Department of Obstetrics and Gynaecology (Monash University, Australia). These samples were stored at -20°C.

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