# STUDIES ON STEROID METABOLISM IN BREAST ADIPOSE TISSUE

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## DEDICATION

With gratitude for their patient tolerance, encouragement and faith in me I wish to dedicate this thesis to my wife Kristine, and my children Robert and Mari.

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## Abbreviations used in text

| | |
|---|---|
| cyclic AMP | Cyclic Adenosine Monophosphate |
| cpm | counts per minute |
| dpm | disintegrations per minute |
| HAN | hyperplastic alveolar nodules |
| PUFA | polyunsaturated fatty acid |
| P/S ratio | ratio of polyunsaturated to saturated fatty acids |
| $^{3}H$ | Tritium |
| 17BHSD | 17-beta hydroxysteroid dehydrogenase |

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## SUMMARY

Adipose tissue is an important site of peripheral oestrogen biosynthesis. Since oestrogens have been implicated in the natural history of breast cancer and the human breast is invested in an abundance of adipose tissue the significance of such activity in breast adipose tissue was investigated.

A rapid, sensitive assay was developed for measuring both aromatase (oestrogen synthesis) and 17-beta hydroxysteroid dehydrogenase (17BHSD), - a pivotal enzyme in the interconversion of both androgens and oestrogens.

Particulate fractions from adipose tissue were incubated with [1-beta $^{3}H$] androstenedione. Aromatase activity was monitored by the release of [$^{3}H$] $H_2O$, 17BHSD activity by measuring formation of [$^{3}H$] testosterone.

All samples investigated possessed both aromatase and 17BHSD activity. Aromatase activity in breast adipose tissue from the cancer patients was significantly higher ($p< 0.0001$) than in the benign group In contrast there was no difference between the groups in the levels of 17BHSD activity.

In 12 cancer patients, steroid metabolism was studied in the quadrants of the mastectomy specimens. Up to five-fold differences in levels of aromatase were observed between different quadrants but there was a significant trend towards higher aromatase activity in the Upper Outer Quadrant ($p< 0.05$). However a much more

significant distribution of activity was apparent in relation to tumour location, the main tumour mass always being located in the quadrant with the highest aromatase activity. In contrast quadrants with the lowest activity never contained tumour foci ($p< 0.001$). The distribution of 17BHSD activity between the quadrants failed to reveal any link with the anatomical location of the quadrant or with tumour site.

Levels of 17BHSD activity were significantly higher around tumours >3cm in diameter compared with smaller tumours ($p< 0.01$). Activity was also higher in patients with nodal metastases compared with those who were node negative ($p< 0.01$). In patients who had received systemic therapy prior to surgery, higher levels of activity were detected in patients who failed to respond to treatment than those who responded ($p < 0.02$). There was no apparent relationship between aromatase activity and tumour size, nodal metastases or response to treatment.

The composition of triglyceride free fatty acids were also measured in twelve patients. A significant inverse correlation was found between the concentration of polyunsaturated fatty acids and adipose tissue 17BHSD activity, but not with aromatase activity. Local levels of polyunsaturated fatty acids may therefore be involved in regulation of 17BHSD activity in adipose tissue.

These results suggest that local aromatase activity could be important in the evolution of breast cancers and that tumours with a poor prognosis are associated with elevated levels of 17BHSD in surrounding adipose tissue. Local steroid metabolism in breast

adipose tissue may therefore be a contributary factor in the natural history of breast cancer.

## Chapter 1.

### Breast Adipose tissue and breast cancer.

"One anatomical feature of the acinar and ductal epithelium of the breast seems to distinguish it from that of nearly all other glandular structures of the body - i.e. from puberty it is invested in an abundance of adipose tissue."

Beer & Billingham. 1978, [7]

The aetiology and natural history of breast cancer have been the subject of numerous investigations over the years[97,155], and although much information has accrued on these subjects we are still a long way from full understanding of this complex and deadly disease. In the paper quoted above, the authors noted that little if any attention has focussed on a fundamental feature of the breast that makes it almost unique in comparison with all other glands in the body, and suggested that adipose tissue could be a significant factor in the aetiology of breast cancer.

In the following account the evidence that adipose tissue is involved in the normal and abnormal development of the mammary gland will first be reviewed before considering whether quantitative or qualitative changes in breast adipose tissue could be linked with breast cancer.

In prepubertal animals and humans a pad of fibro-fatty tissue is laid down in the mammary area and the mammary gland develops from ingrowth of primitive ducts into this fat pad[134]. This growth is stimulated by a combination of hormones in which oestrogen appears to be vital[27,95,125].

The importance of the fat pad in this development was first demonstrated in mice by Meyers in 1919 when he showed that duct growth could be retarded if the size of the mammary fat pad was reduced by food restriction[104]. Hoshino took this a stage further by establishing that duct growth could be completely abolished if the primitive ductal tissue was transplanted away from adipose tissue[82,83].

In fact mammary fat pads do not appear to differ from adipose tissue elsewhere in the body in facilitating ductal growth since transplanted glands are able to grow and develop normally even to the extent of lactation in sites such as the retroauricular fat pad and the peri-renal fat pad[82].

The extent of duct growth in the gland appears to be limited only by the amount of adipose tissue available, and in this respect there seems to be an interaction between the growing ducts and adipose tissue to control the final density of mature ducts[44]. Although the duct system extends to the limits of the fat pad, only a small proportion of the total fat pad volume is occupied and ducts do not touch each other except at points of origin. It appears that around each duct there is a cylinder of adipose tissue into which adjacent ducts do not enter. A similar duct free zone exists at the border of the fat pad. These observations led Faulkin to suggest that the spacing of the ducts in a normal virginal gland is determined by a growth inhibiting system imparted to the surrounding adipose tissue by the existing ducts[44].

From these established ducts, lobules composed of terminal ducts and alveoli arise. In women this occurs some time after puberty[102,144], but in mice it is delayed until the first pregnancy[44,144]. The manner and extent of development nevertheless seems to be similar in both species. The lobules arise from the pre-existing ducts to occupy the previously unoccupied interductal spaces and begin to replace the fatty stroma. If distinct duct growth-inhibition does exist there could

still be several possible explanations for this phenomenon: 1)Terminal ducts could be less sensitive to growth inhibition than larger ducts, and alveoli could be even less sensitive so that secreting alveoli can come into contact with one another; 2)Mammogenic hormones which stimulate lobule growth alter the growth-inhibiting mechanism; or 3)The inhibition only applies to ductal growth and only the smaller ducts can give rise to alveoli (i.e. alveoli cannot give rise to alveoli) then the number of alveoli is limited to those that can cover the terminal ducts and thus the whole mammary structure could be regulated by regulating the ducts.

Evidence that adipose tissue could be important in the development of mammary tumours comes from further transplantation studies in mice. Murine mammary tumours are thought to develop from premalignant hyperplastic growths known as hyperplastic alveolar nodules (HAN)[30]. The importance of adipose tissue in the growth of these HAN and in their transformation into malignant tumours was demonstrated when DeOme showed that 9 of 19 HAN grew and developed into malignant tumours after transplantation into adipose tissue, whereas very little growth occured in 60 HAN transplanted subcutaneously (remote from adipose tissue) where only one tumour evolved[31]. Interestingly no growth was observed in HAN transplanted into intact mammary glands suggesting that HAN are still susceptible to the growth regulating mechanisms in the normal gland[44]. Such regulating mechanisms may also inhibit tumour growth since it was also noted that transplanted tumours grew more slowly in intact mammary glands than in adipose tissue cleared of glandular elements[44].

In summary therefore, the evidence from mice indicates that adipose tissue is essential for normal mammary gland development, for the growth of hyperplastic nodules and for the development of tumours from these nodules. Adipose tissue associated with ducts in the normally developed gland however, seems to be able to inhibit further duct growth, retard the growth of transplanted hyperplastic nodules and impede the growth of transplanted tumours.

If similar mechanisms operate in humans, the normally developed breast may have an optimum adipose/glandular tissue ratio which is achieved at the completion of ductal growth and maintenance of this ratio should inhibit growth of hyperplastic lesions. For hyperplastic lesions to develop in a mature breast there must therefore either be a relative or absolute increase in the amount of adipose tissue present to permit or stimulate its growth, or a loss of the growth-inhibitory process. If the latter occurs, the mechanism for bypassing the inhibition must lie within the adipose tissue surrounding the HAN rather than within the HAN itself since the HAN loses its ability to grow when it is transplanted into an intact mammary gland[44].

Comparison of mammary gland structure in different species in relation to their mammary cancer rates supports the suggestion that mammary adipose tissue is involved in tumour development.

Mammary cancer only seems to occur to any great extent in humans[38], cats[41], dogs[41,140], rats[86] and mice[144]. Ruminants such as cows and goats, although bred for their large udder size appear to be

resistant to the disease[119,127]. All of these species have similar mammary gland structure and function at the level of ducts and alveoli; the only substantial difference between them being the amount of adipose tissue distributed in the gland. The mammary gland of ruminants mainly consists of alveoli which closely abut one another without extensive quantities of intervening tissue: adipose tissue is found next to the skin and between the gland and the abdominal wall but is not heavily interspersed between alveoli. In contrast the species which are susceptible to mammary cancer all have adipose tissue occupying interalveolar spaces[119]. It is interesting that these species are all monogastric and frequently ingest considerable quantities of lipid resulting in intermittently high blood lipid levels whereas ruminants never experience significant elevation of blood lipids, largely due to their low fat diet and complex digestive systems[119]. (The importance of this factor in producing mammary gland adiposity was demonstrated by Stewart who induced adipose tissue deposition in a cow's udder by an intravenous infusion of a lipid emulsion[150].) It is not only ruminants however, which have reduced mammary adipose tissue; rabbits, although monogastric, have very little mammary adipose tissue and a low incidence of mammary cancer[119,144].

A further feature which distinguishes humans from other species is that the human breast undergoes major anatomical development at the time of puberty[152]. Breast enlargement is in fact the first external sign of impending puberty in girls[102], and the breast can achieve its adult size before the first ovulation, which is usually about a year or two after menarche[143,152]. Much of this pubertal breast growth is due to the development of the fatty stroma[134] and

these tissues will predominate until the first pregnancy.

Since evolution has always operated to maximise fertility, in most species pregnancy usually follows immediately after puberty and the mammary gland becomes a functional organ soon after the commencement of an animal's reproductive life. It is interesting that man has chosen to suppress reproductive potential in only a few selected species: his wife, his domestic pets the cat and dog, and his laboratory animals - the species which now develop breast cancer. In his farm animals, on the other hand, fertility has always been at a premium in order to achieve maximum production of milk or offspring resulting in little if any latent period between mammary gland development and function.

Delay in initiating normal reproduction appears to be a critical factor in determining mammary cancer risk since it has now been established that an early first pregnancy does appear to protect humans[98], and animals[4,86,112,144,157] from subsequent tumour development. A possible explanation for this phenomenon is that the development of mammary epithelial cells for lactation is thought to be a terminal process, i.e. once the cell achieves the differentiated state of lactation it will never perform any other function[111,133]. Undifferentiated "stem" cells in the gland are thought to be the cells which are most likely to undergo malignant transformation[111,155], and an increase in the time between breast development and functional differentiation of the gland will increase the period during which large numbers of undifferentiated cells are available for transformation. It may also be important that during this period there is a considerable amount of adipose

stroma present in the gland and while it is likely that the crucial effect of pregnancy is to induce differentiation of stem cells it could be significant that pregnancy and lactation result in a marked reduction in the proportion of adipose tissue in the breast[134].

There are therefore a number of possible factors which may contribute to the varying incidence of mammary cancer between species but the evidence does support the theory that tumours develop in glands containing adipose tissue. What then is the evidence that an increase in the amount of adipose tissue is associated with increased risk?

The risk of developing mammary cancer increases in both humans[38] and animals[41,139] with increasing age. In Western countries the increase in human breast cancer is almost linear with age[32], and this correlates well with the proportion of adipose tissue within the breast which also increases with age due to a gradual atrophy of the glandular structures[126].

Apart from ageing, the only other established condition which increases the amount of adipose tissue in the breast is obesity[151]. In mice Waxler has shown that obesity appears to accelerate tumour development[161]. It seems likely that similar mechanisms could operate in humans since epidemiological studies[34,35,170] have shown an increased incidence of breast cancer in obese women with the difference being most marked in older women. Conversely there is also evidence that women who have

reduced amounts of body fat (athletes and swimmers) have a decreased incidence of breast cancer[64]. Finally, if adipose tissue does favour the growth of tumours then one would expect obese women or women with predominantly fatty breasts to have more rapidly growing tumours. While tumour growth rates have not been studied in relation to breast type the reports[33,39,40] of a poorer prognosis in obese breast cancer patients is consistent with more rapid tumour growth in these women.

Precise measurement of the amount of adipose tissue in breasts is extremely difficult, if not impossible in vivo, and it is not surprising therefore that comparison of the amounts of adipose tissue present in cancerous breasts and controls have not been reported. Wynder, however, reports that Japanese women with breast cancer have larger breasts with more subcutaneous fat than women without the disease[169]. Although larger breasts might be expected to contain more adipose tissue, breast volume is apparently not a reliable guide to the amount of fat in the breast and gives no indication of the likely adipose/glandular tissue ratio within the organ[151].

The fact that a difference was found in Japanese women is interesting since the lowest incidence of human breast cancer is found in indigenous Japanese women[38]. This incidence has however been rising in recent years and a marked increase is seen in Japanese women who have emigrated to the West[24,42]. Sasano[135,136] has suggested that this could be linked to an increase in the amount of breast adipose tissue: In an analysis of

breasts removed at autopsy from a series of indigenous Japanese women and Japanese immigrants to Hawaii, the incidence of hyperplastic breast disease was 51.4% in the Hawaiian Nissei compared to only 18.7% in the indigenous population, with a positive correlation between the presence of hyperplastic disease and the amount of breast fat. The Hawaiian women had significantly larger breasts than the native Japanese which was entirely due to an increase in adipose tissue.

The predominance of adipose tissue in cancerous breasts has been demonstrated by Anastassiades[3] who analysed the distribution of solid and fatty tissues in a series of 112 cancerous breasts and found that 94(86%) of the breasts were entirely or predominantly fatty. Even the 18 cases with a reasonable amount of "solid" tissues still contained considerable quantities of adipose tissue.

The above evidence therefore supports the theory that adipose tissue is a factor in the development of hyperplastic breast disease and breast cancer but the precise role that it plays has yet to be determined. Since adipose tissue is vital for the normal growth of the mammary gland it is reasonable to suggest that it produces or processes growth-stimulating factors which act locally on epithelial cells. Moreover since cell multiplication is an essential step in the development of premalignant or malignant lesions it seems likely that the same mechanism could act as a promoter of tumour development.

Although the precise nature of growth-stimulating factors which act

on the breast is still the subject of much debate, it is accepted that an essential requirement for normal development is a suitable hormonal environment[27,95,137]. Various combinations of the hormones oestrogen, progesterone, prolactin, growth hormone and corticosteroids are needed to produce optimum growth of ducts, development of lobules and alveoli, and differentiation for lactation[6,95,125,137]. Although with such a complex inter-play of hormones it is difficult to single out one factor, current dogma favours oestrogen as the key hormone in the growth and development of the normal ducts[95,137]. In keeping with this, it has been shown that if breast tissue is maintained in organ culture, as gland explants, oestrogen is capable of stimulating nucleic acid synthesis in breast epithelial cells[158]. In recent cell culture experiments however, Haslam has demonstrated that oestrogen is only capable of stimulating epithelial cell growth when stromal fibroblasts are present, either in large numbers or in direct contact with the epithelial cells[74]. When isolated epithelial cells and fibroblasts were cultured together, differences occured in the morphology of both cell types, suggesting that cell-cell communication or another interactive phenomenon was taking place and that it was bi-directional. Furthermore as epithelial cells became confluent in co-culture, the presence of live fibroblasts appeared to inhibit epithelial cell proliferation - provided there was no added oestrogen.

Such observations are interesting in view of the relationship which has already been outlined between the mammary fat pad and the developing gland. The suggestion that local levels of oestrogen could be the factor that determines whether stromal cells will

stimulate or inhibit the growth of epithelial cells is particularly intriguing since excessive oestrogenic stimulation of the mammary gland has been suggested as a key factor in mammary tumour development[90]. The whole issue is now also of particular relevance to the role of adipose tissue within the gland since it has now been established that adipose tissue is capable of synthesizing oestrogens locally from circulating adrenal androgens[2,10,29,37,53,55,57,65,115,121,138,147,148,171].

Although it is only in the past few years that attention has focussed on the possible significance of oestrogen biosynthesis in adipose tissue, compelling evidence in support of its role was provided, quite unintentionally, by the experiments of Waxler in the 1950's[161-163]. Gold thioglucose, which destroys the satiety centre of the hypothalamus, was administered to mice to induce obesity. Mammary tumours developed earlier in the obese mice than in non-obese controls[161]. A group of the obese animals were then given a reducing diet. Obese animals which were slimmed down to the same weight as controls then took much longer than the controls to develop mammary tumours[162]. No satisfactory explanation was offered for this unexpected finding.

Some years later, however, additional studies using this experimental model revealed that the animals which became obese also became infertile and remained infertile even when slimmed down to their original weight[163]. Investigation of these animals revealed that they all had atrophic ovaries which presumably resulted from coincidental damage to the hypothalamic centre which normally releases gonadotrophins - a rather unusual form of

hormonal ablation. Since ovarian ablation is known to markedly reduce the incidence of mammary cancer[41,45,92,139] - the lack of ovarian function in these animals presumably accounts for the fall in tumour incidence in the "slimmed" animals.

Despite the lack of functioning ovaries in the obese animals, vaginal smears indicated that chronic oestrogenic stimulation was occuring. Waxler at that time did not speculate as to the likely source of this oestrogen but the fact that there was no evidence of oestrogenic stimulation in the "slimmed" animals suggests that it was being produced in adipose tissue. The implication from these experiments that adipose tissue is able to produce sufficient oestrogen to enhance tumour growth in the absence of functioning ovaries lends strong support to the theory that local oestrogen production could be an important factor in the development of mammary tumours.

Although these experiments were performed in the 1950's it was not until 1972 that definitive proof of the ability of adipose tissue to produce oestrogens appeared[138]. This early study detected the formation of oestrone from the androgen, androstenedione. This, and the convertion of testosterone to oestradiol have now been confirmed in adipose tissue from a number of sites[2,10,29,37,53,55,57,65,115,121,147,148,171]. The reaction involves the formation of an aromatic ring in the A-ring of the steroid nucleus; the process is therefore known as aromatisation and the enzymes which perform the reaction are known as aromatase(s)[49].

Peripheral (extra-gonadal) aromatase activity has also been shown to reside in skin[12], muscle[94], liver[65,149], hair-follicles[141], and brain[113,114]. In these tissues and in adipose tissue, activity is located predominantly in the stromal cells[2,29,147], and while it is generally accepted that peripheral aromatase is the major source of oestrogen production in post-menopausal women[69] little is known about the factors which influence levels of activity in vivo. Levels of activity vary between individuals and also between different body sites[37,53,55,57,65,115,121,138]. Possibly as a result of this variation there is little consensus about the relationship of activity to such factors as menopausal status, age and obesity.

MacDonald[96] was unable to demonstrate any difference in activity in adipose tissue from ovulatory or anovulatory young women, but suggested that higher activity was present in postmenopausal women. This is in agreement with the findings of Cleland[28], Forney[57] and Hemsell[76] who suggest that activity increases with age. Deslyspere[37] however, has recently published results indicating that activity is greater in premenopausal women, and other studies have failed to find any correlation between activity and age[10,171]. Edman[43] appears to be the only one who has found that activity per unit mass of adipose tissue increases with obesity, but there is little doubt that total peripheral aromatase is increased in obese subjects compared with controls[89,173].

Adipose tissue can also hydrolyse oestrone sulphate to produce free oestrone[72] and can interconvert oestrone and oestradiol via the 17-beta-hydroxysteroid oxidoreductase pathway[10,11,37,52,55]. This

activity could be important since oestradiol is biologically much more active than either oestrone or its sulphate[50,71,72].

The biological significance of oestrogen biosynthesis in adipose tissue can be inferred from the association of obesity with oestrogenic effects. These effects are apparent from an early age: Frisch[62,63] has demonstrated that the onset of menarche, (which is determined by oestrogen levels[22]), is related to body weight and that the weight/height ratio appears to be the critical factor. This was confirmed by Zacharias[172] who also showed that menarche occurs significantly earlier in obese girls. This acceleration of maturation by obesity in girls was again noted in the Bogalusa Heart Study[160] where it contrasted with the effect on boys. Obese boys were prone to develop gynaecomastia and when this occurred it was associated with a delay in male sexual maturation. Both of these features would be consistent with an oestrogenic effect of obesity. Such an antagonistic effect of obesity on male sexual maturation has also been observed in rats: those animals with the lowest percentage of body fat having significantly larger prostates, seminal vesicles and testes than fatter controls[66].

Although these studies did not involve correlation of levels of oestrogen with obesity, other investigations have established this link. DeWaard[36] has shown that in obese postmenopausal women there is a significant link between levels of oestrone excretion and obesity and that this correlation disappears after weight loss. He was unable to demonstrate any effect on levels of oestradiol, probably because the major circulating oestrogens in postmenopausal

women are oestrone and its sulphate. O'Dea[118] however, has reported decreased serum oestradiol levels in a group of women who lost a greater amount of weight.

Kirschner[89] reported similar findings in both men and women and noted that in obesity there is increased production of androstenedione which contributes to the elevated oestrogen production. In addition he and others[67,89,146] have noted that obesity is associated with decreased levels of sex-hormone-binding-globulin which results in higher levels of free (unbound) oestrogen in plasma. (Unbound oestrogens are believed to be the biologically active mediators of hormone action[5,146]). Not only is adipose tissue a significant source of oestrogen production, it is also an important site for the uptake of oestrogens from plasma[37,46,47,173] resulting in local concentrations of oestrogen which may be up to fifteen times higher than in plasma[37].

Evidence that such altered sex-hormone metabolism in obesity could have an effect on tumour biology comes from the well-documented association of obesity with tumours of the endometrium, prostate and breast[89] - organs which are normally targets for hormone action. Furthermore, DeWaard has found that oestrogen-receptor positive breast cancers are more common in obese patients than in leaner controls[33].

In summary therefore there is evidence to support the theory that adipose tissue plays a vital role in the normal development of the mammary gland and that the presence of excess adipose tissue is

associated with the development of hyperplastic lesions and malignant tumours. Recent evidence suggests that the stroma may interact with epithelial cells to stimulate or inhibit growth and that a crucial factor in determining the nature of this interaction could be levels of oestrogen. Since adipose tissue is capable of synthesizing oestrogen locally within the breast it is not unreasonable to suggest that an increase in oestrogen biosynthesis within the breast could favour the development of breast cancer.

Despite the above evidence very few studies have been performed on breast adipose tissue to establish if there are differences in oestrogen biosynthesis between cancer cases and controls. Nimrod and Ryan[115] compared aromatase activity in adipose tissue from five cancerous breasts with two controls and concluded that they had similar activity - but one of their control samples came from the uninvolved breast of a cancer patient. Perel & Killinger[121] compared five cancer cases with four controls and concluded that there was no statistically significant difference between the groups - although they did find the highest levels of activity in the cancerous breasts. Beranek[10] compared 17 cancer cases with 16 controls and also concluded that there was no statistically significant difference in levels of activity between the two groups, but he was only able to detect activity in 50% of his control group whereas activity was detected in 82% of the cancer cases, suggesting that with a more sensitive assay a significant difference might have emerged.

The aim of this project was therefore to establish a sensitive assay for measuring oestrogen biosynthesis in breast adipose tissue

in order to undertake detailed investigation of levels of activity in cancerous and non-cancerous breasts.

# Chapter 2.

## Development of aromatase assay.

The conversion of androgens to oestrogens is catalysed by the aromatase complex and involves the loss of the $C_{19}$ angular methyl group and cis elimination of the 1-beta and 2-beta hydrogens from the steroid nucleus of the precursor so that the A ring becomes aromatic[49]. Androstenedione and testosterone are thus converted to oestrone and oestradiol respectively as is shown in Figure 2:1.

Conventional assays for aromatisation use androgens labeled with tritium at a stable position in the steroid nucleus (eg $C_7$ which is rarely metabolised) and involve the tracing of the label into oestrogen products[10,109,115,138]. This requires the chromatographic separation of oestrogens from other steroid components which are likely to be radioactively labeled i.e. the unmetabolised androgen precursor and other androgen products. Such purifications can be both difficult and time consuming. Since it is planned to conduct a large survey of enzyme activities in breast adipose tissue, a more rapid aromatase assay is desirable.

Such a rapid assay has been described which uses androgen substrates labeled in the 1-beta or 1-beta & 2-beta positions and measure the release of tritium as water or aqueous products during the formation of the aromatic A ring[12,61,156]. Most of the studies using such assays have either been on very active tissue preparations such as placenta[156] or have used placental preparations to validate the assay before applying it to less active tissues such as adipose tissue stromal

OESTROGEN BIOSYNTHESIS

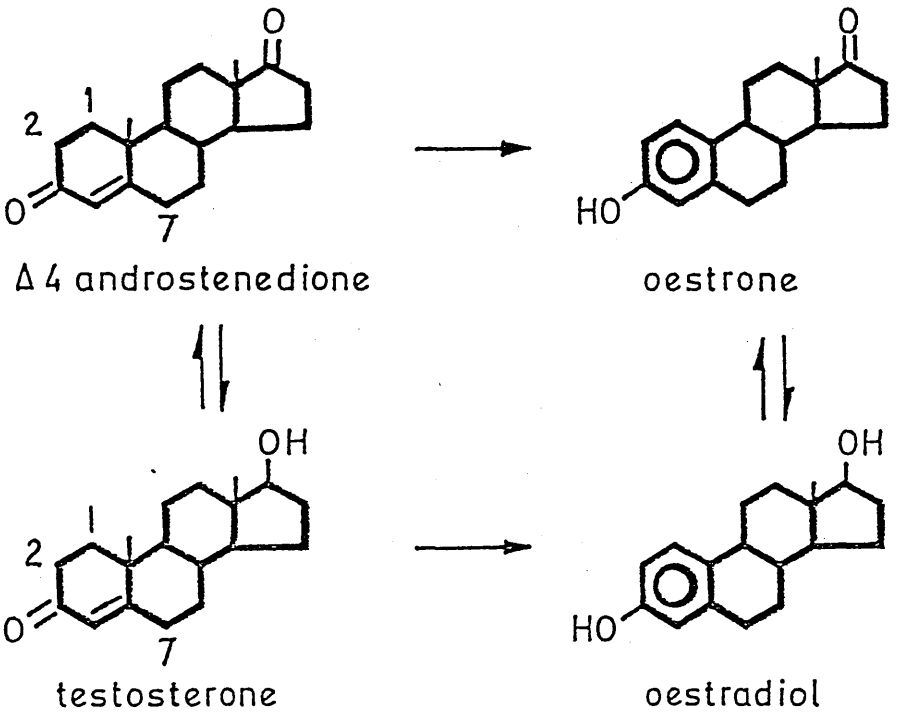



Figure 2:1. Oestrogen Biosynthesis.

The $C_1$,$C_2$ and $C_7$ positions on the steroids are indicated

cells[2,28,29,103,147,148]. Since all studies to date on adipose tissue have found comparatively low aromatase activity (less than 1% convertion of androgen[2,10,29,37,53,55,57,65,115,121,138,147,148]), it is critical to establish the sensitivity and reproducibility of the "tritium release" assay in adipose tissue initially before embarking on a large scale study. The immediate objectives of this project were therefore to establish whether the rapid "tritium release" assay will accurately measure the extent of oestrogen biosynthesis in adipose tissue and to define the form of tissue preparation and assay conditions which will produce optimum results.

The "product identification" assay[109] against which the "tritium release" assay will be compared is described below (page 40). This assay has been in use for a number of years and its accuracy and reliability are well established[108,109].

A variety of tissue preparations have been used to measure adipose tissue aromatase activity in vitro. These include tissue slices[57], minces[57], cell suspensions[13,53] or cultures[2,121], homogenates[10,13,138] and various subcellular fractions[13,57,115]. Opinions differ on which is the most suitable. The method described by Nimrod & Ryan[115] utilised the soluble extract of tissue homogenates and has subsequently been used successfully by a number of groups. This method was therefore adopted for the initial experiments.

A variety of modifications have been suggested for the "tritium release" assay which was originally described by Frieden et al[61].

A modification of this method has been used with success in The Department of Clinical Surgery of Edinburgh University for studies on placental aromatase. This method (page 39) was therefore adopted for the initial comparisons and modified as the experiments progressed.

A large number of experiments were performed in the initial stages of this project in order to establish an accurate, reproducible assay. Only the most relevant of the experiments are included in the following sections. As improvements were made they were incorporated into the method for subsequent experiments. To avoid repetition of the full assay procedure after each modification, details will be given only of the particular feature of the assay which is under investigation.

Adipose tissue was used in the majority of the experiments. In some of the preliminary experiments ovarian microsomes were used since they usually exhibit higher aromatase activity than adipose tissue and therefore require shorter incubation times.

## Experiment 1.

## Comparison of the "product identification" and "tritium release" assays for measuring aromatase activity in breast adipose tissue.

### Materials & Methods

#### Chemicals:

1-beta,2-beta $^{3}H$ androstenedione (56 Ci/mmol) and 1,2,6,7 $^{3}H$ androstenedione (83 Ci/mmol) were obtained from New England Nuclear. Prior to use they were purified by silica gel thin layer chromatography in chloroform:acetone (185:15 v/v).

Radio-inert androstenedione, glucose 6 phosphate dehydrogenase (Type XI), NAD (grade V), glucose 6 phosphate (disodium salt), ATP (Disodium salt), NADP (Monosodium salt), nicotinamide and activated charcoal were obtained from Sigma Chemical Co Ltd, Poole, Dorset. NE260 Scintillant was obtained from Nuclear Enterprises Ltd, Edinburgh; Scintol 7 from Koch-Light Ltd, Haverhill, Suffolk; and bovine serum albumin from Armour Pharmaceuticals, Eastbourne. Other chemicals and solvents were obtained from BDH Chemicals Ltd, Poole.

#### Patients:

Adipose tissue was obtained from breast cancer patients treated either by mastectomy or wide local excision.

Tissues:

Breast adipose tissue was obtained from the surgical specimens immediately after excision and transferred to the laboratory on ice. All procedures were then performed at $4^{o}C$ unless otherwise stated. Samples were rinsed in 0.1M phosphate buffer (pH 7.4) before carefully dissecting adipose tissue free from any obvious breast parenchyma or fibrous tissue.

Preparation of Soluble Fraction of Adipose Tissue:

Tissue was processed according to the method of Nimrod & Ryan[115]. Two grams of adipose tissue were homogenised by hand in a glass-to-glass homogenizer with 2 ml of 0.1M phosphate buffer (pH 7.4) containing nicotinamide (10mM) and Magnesium Chloride (5mM) then centrifuged at 800g for 5 minutes. The resultant supernatant (soluble fraction) was separated from the upper layer of solid lipid and the lower layer of cell debris using a pasteur pipette.

Assays:

2 grams of adipose tissue obtained from patient 1 was used to prepare 2ml of "soluble extract". 0.5ml was then incubated according to the "tritium release" method outlined below, 1ml was incubated in parallel by the "product identification" method (below).

Tritium Release Assay

Cofactors [glucose 6-phosphate (10mM), glucose 6-phosphate dehydrogenase (2u/ml) and 2 mM each of NAD, NADP and ATP] and substrate [100 nM 1-beta,2-beta $^{3}H$ androstenedione (2.5 uCi) 2.5 x $10^{6}$ dpm) ] were pre-incubated for 15 minutes at 37°C in a total volume of 500 ul phosphate buffer (0.1M, pH 7.4). Enzyme reactions were started by addition of the soluble fraction (500 ul) to substrate and co-factors. A blank incubation was also set up using 500 ul of bovine serum albumin (2.0 mg/ml) in place of the soluble fraction. The incubation was then performed over 3 hours at 37°C with continuous shaking. Aliquots (500 ul) of the reaction mixture were removed into 1.625% charcoal solution (800 ul) immediately and after 3 hours, mixed, and allowed to stand on ice for a further 20 minutes with occasional further mixing to adsorb the steroids onto the charcoal. The charcoal was then precipitated by centrifugation at 2,000 g for 15 minutes and the supernatant decanted into a glass counting vial containing NE 260 Scintillation fluid (10 ml). Radioactivity was measured on a Packard Tri-Carb Liquid Scintillation spectrometer. Counts obtained in the blank incubations were subtracted from the counts in the tissue incubation to determine the amount of tritium released during aromatisation.

The % conversion of androstenedione to oestrogen was calculated according to the formula:

$$\frac{^{3}H \text{ released as water (dpm)}}{^{3}H \text{ added as androstenedione}} \times 100$$

Product Identification Assay

An assay system was set up as for the tritium release assay but using 1,2,6,7 $^{3}H$ androstenedione (100 nm, 50 uCi, $_{2}50 \times 10^{6}$ dpm) as substrate in a total volume of 2ml (cofactors and substrate in 1ml + 1ml of the soluble tissue fraction). The reaction was terminated after three hours by addition of methanol (2ml). Radioinert oestrone and oestradiol (500ug) were added to monitor manipulative losses.

The scheme used for the purification of oestrone / oestradiol and determination of their specific activity is outlined in Figure 2:2.

Steroids were extracted twice with ethyl acetate (5ml) and the ethyl acetate layers were taken off, bulked and evaporated to dryness. The residue was dissolved in 4ml ether:petroleum ether (40-60$^{o}$C) [1:1 v/v] and partitioned with 1N. sodium hydroxide (1ml) three times. The ether:pet ether fraction was washed once with water (1ml) and the water combined with the sodium hydroxide extract. The sodium hydroxide extract was neutralised with sodium bicarbonate (0.56g) and re-extracted twice with ethyl acetate (2ml). The ethyl acetate fraction was evaporated to dryness, dissolved in ethanol (0.2ml) and spotted onto a silica gel TLC plate and run in chloroform:acetone (98:2 v/v) for three hours with oestrone and oestradiol standards. Oestrone and oestradiol fractions were visualised under u.v. light (375nm) and the appropriate areas of the plates scraped off and eluted with ethanol after deactivation of the silica gel with a drop of distilled water. From this point the oestrone and oestradiol fractions were

Purification and specific activity determination of oestrone / oestradiol

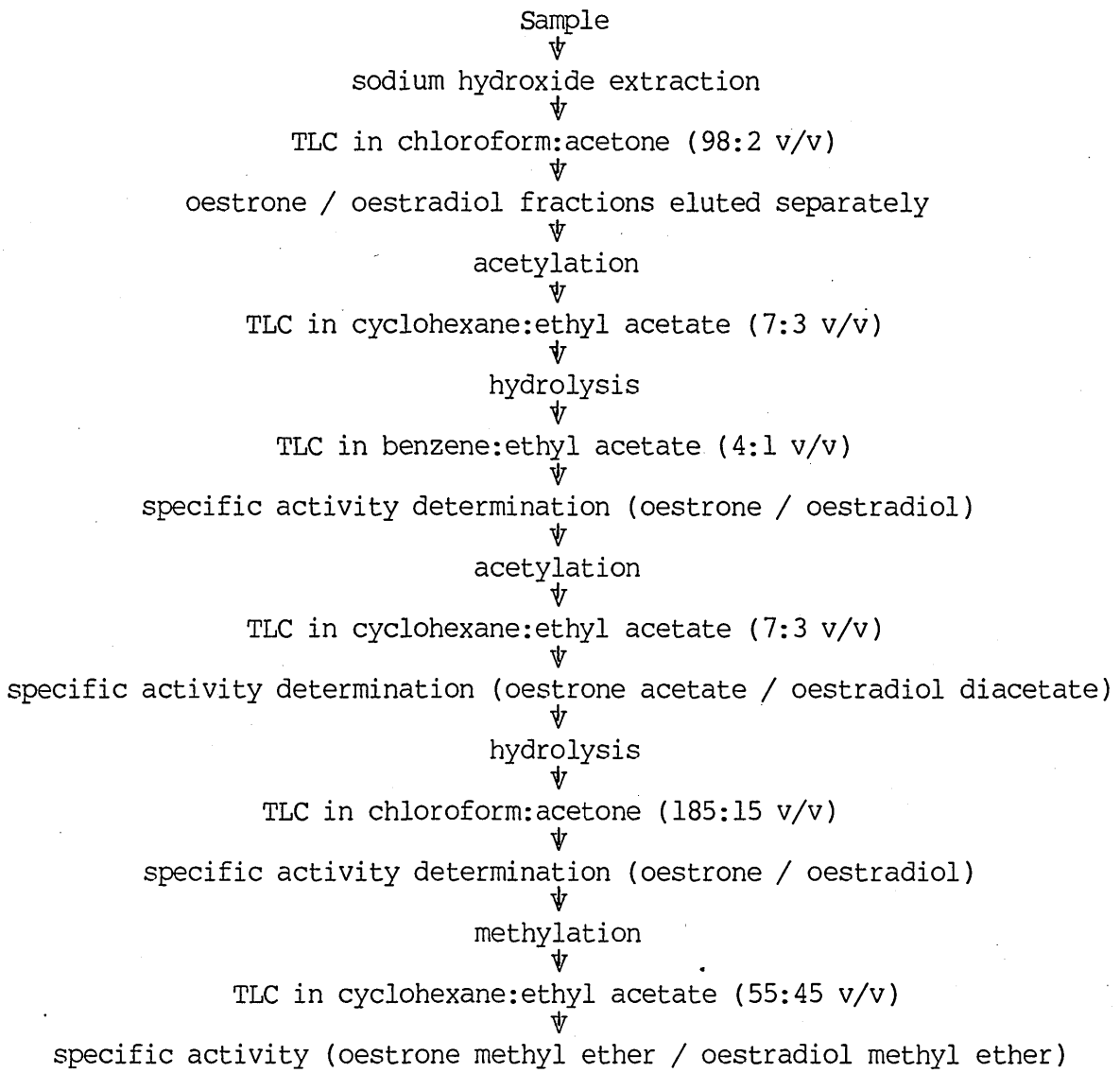



Figure 2:2.

PRODUCT IDENTIFICATION ASSAY

purified and characterised separately to determine their specific activities.

The ethanol was evaporated to dryness and the oestrogens acetylated by adding acetic anhydride (6 drops) and pyridine (3 drops) and incubating at 60°C for 1 hour. Methanol (1ml) was added, evaporated to dryness and the residue spotted onto a silica gel plate and run in cyclohexane:ethyl acetate (7:3 v/v) for 1 hour with appropriate standards. Oestrone acetate / oestradiol diacetate were visualised under u.v. light (280nm), scraped off, eluted with ethanol and dried down.

The acetates were then hydrolysed by dissolving in methanol (1ml), adding potassium carbonate (0.25ml, 2%) and incubating overnight at 37°C. Distilled water (3ml) was then added and the steroid extracted twice with ethyl acetate (5ml) and evaporated to dryness. The residue was spotted onto a silica gel plate and run in benzene:ethyl acetate (4:1 v/v) for 1 hour with appropriate standards (oestrone + oestrone acetate or oestradiol + oestradiol diacetate). The free steroids were visualised and eluted as before.

The specific radioactivity of the fractions were determined at this stage by measuring both the radioactivity and the total oestrone / oestradiol associated with the fractions. The steroids were dissolved in ethanol (5ml). $^{3}H$ was determined by liquid scintillation counting of an aliquot (1/10) in Scintol 7 (10 ml). Total oestrone / oestradiol was measured by comparing u.v. absorption (280 nm) on a Unicam SP800 spectrophotometer with the

optical densities of authentic oestrone / oestradiol standards.

The free steroids were then acetylated and chromatographed as before. Oestrone acetate / oestradiol diacetate was eluted and its specific activity determined. $^{3}H$ was determined by liquid scintillation counting of an aliquot (1/5) in Scintol 7 (10ml). The acetates were measured by Gas Liquid Chromatography of an aliquot (1/10) on a five foot column of 3% OV1 on Gas Chrom Q (100 - 120 mesh - Applied Science Laboratories Inc.) at 223$^{o}$C. Testosterone acetate (10ug) was used as an internal standard.

The acetates were then hydrolysed and chromatographed as before. The free steroids were eluted and their specific radioactivity determined as previously using an aliquot (1/4) for liquid scintillation counting and measuring total oestrone / oestradiol by u.v. absorption.

The free steroids were then dried and methylated by the addition of distilled water (10ml), boric acid (0.18g), sodium hydroxide (0.8ml, 20%) and dimethyl sulphate (0.2ml), and incubation for 30mins at 37$^{o}$C. A further 0.4ml of 20% sodium hydroxide and 0.2ml of dimethyl sulphate were added and the mixture incubated for a further 30 minutes. Sodium hydroxide (2ml, 20%) and hydrogen peroxide (0.5ml, 30%) were then added and the steroids extracted into petroleum ether (25ml).

The oestrone / oestradiol methyl ethers were chromatographed in cyclohexane:ethyl acetate (55:45 v/v), visualised under u.v. light, eluted and their specific activities determined. $^{3}H$ was

determined by liquid scintillation counting of an aliquot (1/3) in Scintol 7 (10 ml). The methyl ethers were measured by Gas Chromatography of an aliquot (1/5) using testosterone (10ug) as an internal standard (conditions as before).

If the oestrone / oestradiol fractions are pure, the specific activities of the free steroid, the acetate and the methyl ether will be constant. Purification by chromatography and chemical derivative formation continued until the three specific activities differed by less than 10%.

The % conversion of androstenedione (A) to oestrone ($E_1$) was calculated as follows:

$$\frac{^3H \text{ in } E_1 \text{ recovered} \times \text{cold } E_1 \text{ added} \times 100}{^3H \text{ in A added} \times \text{cold } E_1 \text{ recovered}}$$

which may be simplified to

$$\frac{\text{Specific radioactivity of } E_1 \text{ recovered} \times \text{cold } E_1 \text{ added} \times 100}{^3H \text{ in A added}}$$

A similar calculation is used to determine the conversion to oestradiol, and the two figures added to derive the total oestrogen formation.

Results:

These are shown in Table 2:1.

TABLE 2:1.

PRODUCT IDENTIFICATION ASSAY

| STEROID | COLD STEROID (nm) | dpm | Spec. Act. |
|---|---|---|---|
| FREE OESTRADIOL | 970 | 1650 | 1.7* |
| FREE OESTRONE | 936 | 4135 | 4.42 |
| OESTRONE ACETATE | 798 | 3339 | 4.18 |
| FREE OESTRONE | 410 | 1610 | 3.93 |
| OESTRONE METHYL ETHER | 267 | 1150 | 4.31 |

AVERAGE SPECIFIC ACTIVITY (OESTRONE) = 4.21

PERCENTAGE CONVERSION (OESTRONE) = 0.021%

TRITIUM RELEASE ASSAY

| TIME (hrs) | TOTAL dpm BLANK | TISSUE | %CONVERSION |
|---|---|---|---|
| 0 | 54735 | 48658 | |
| 3 | 82035 | 130373 | 1.00 |

* The specific activity of oestradiol at the first estimation was 1.7 equivalent to a % conversion of 0.008. With such a low conversion, no further purification of oestradiol was performed. Even if this activity were maintained throughout the subsequent steps total conversion (ie oestrone + oestradiol) is unlikely to exceed 0.03%.)

Comment:

There is considerable discrepancy between the two assays, with apparently greater activity in the "tritium release assay". There are several possible explanations for this: further metabolism of the formed oestrogens could occur leaving only small amounts of labeled oestrogen to be detected in the "product identification assay" (subsequent experiments however, suggest that further metabolism of oestrogens in 3 hour incubations is negligible). Alternatively, in the tritium release assay there could be non-specific release of tritium into aqueous products or contamination of the aqueous extracts by radiolabeled steroids.

## Modification of the Tritium Release Assay.

A series of modifications were introduced to the tritium release assay to eliminate contamination of the aqueous extracts by steroids, reduce non-specific release of tritium and maximise the detectable aromatase activity.

The modifications which significantly improved the assay are outlined in the following sections.

### Modification 1.

Reduction of steroid contamination of the aqueous fractions by extracting the non-polar steroids into chloroform.

Method:

Incubations were performed with ovarian microsomes as substrate. Extraction procedures were compared as follows:

A. Standard extraction: 2 x 200ul aliquots of incubate each extracted with 800ul of 1.625% activated charcoal (20 mins), samples then centrifuged and supernatants decanted for counting.

B. Modified extraction: 500ul aliquot of incubate dispensed into 3ml of chloroform and thoroughly shaken then centrifuged. 2 x 200ul aliquots were then removed from the aqueous phase and extracted with charcoal as in A above.

Duplicate counts were therefore obtained for each time point. The two were then averaged to calculate the total dpm.

Results: The results are shown in Table 2:2.

Conclusion: Chloroform extraction markedly reduces the levels of radioactivity detected in the aqueous extracts, presumably by reducing steroid contamination.

| | No Chloroform | | | Chloroform | | |
|---|---|---|---|---|---|---|
| TIME | BLANK (dpm) | MICROSOMES (dpm) | % conv. | BLANK (dpm) | MICROSOMES (dpm) | % conv. |
| 0 | 48463 | 65673 | | 24230 | 22740 | |
| 15MINS | 49375 | 350385 | 6.0 | 22933 | 241298 | 4.4 |

TABLE 2:2.

The effect of chloroform extraction on the levels of radioactivity detected in the tritium release assay.

Modification 2.

Increasing the concentration of charcoal in the extractions.

Method:

Two parallel incubations were performed as in 1B above (incorporating the chloroform extraction). In incubation A extraction was performed with 1.625% charcoal, in incubation B with 5% charcoal. Ovarian microsomes were again used as substrate.

Results:

The results are shown in Table 2:3. These reveal a significant reduction in the levels of radioactivity being detected in the aqueous extracts.

To investigate if contamination of the aqueous extracts by radiolabeled steroids was still occuring, a further incubation was performed with one aliquot being dispensed into scintillant and counted as before. The second aliquot was evaporated to dryness under a continuous stream of nitrogen to eliminate any tritiated aqueous products. 1ml of distilled water was then added together with scintillant and the mixture counted in the usual manner.

Evaporation of the duplicate aliquots revealed that there was no longer any significant contamination of the aqueous extracts by labeled steroids.

| | A (1.625% CHARCOAL) | | | B (5% CHARCOAL) | | |
|---|---|---|---|---|---|---|
| TIME | BLANK | MICROSOMES | % | BLANK | MICROSOMES | % |
| 0 | 24650 | 23820 | | 18330 | 17920 | |
| 15mins | 24325 | 240330 | 4.3 | 18650 | 210320 | 3.8 |

TABLE 2:3.

The effect of increased charcoal concentration on the levels of radioactivity detected in the tritium release assay.

Modification 3.

The use of a concentrated "particulate fraction" of adipose tissue.
Prolonging the incubation time.
Reducing the amount of radiolabeled substrate.

Further improvement in the assay required either a reduction in the non-specific release of tritium or a relative increase in the specific tritium release. Non-specific release of tritium should be decreased by reducing the quantity of radiolabeled substrate in each incubation. Specific release of tritium due to oestrogen formation could be enhanced by allowing the reaction to run for a longer time and by including more tissue in each incubation. (Nimrod & Ryan have reported that this can be achieved by precipitating particulate fractions from larger quantities of adipose tissue[115].)

Preparation of Particulate Fraction:

5grams of adipose tissue were homogenised in buffer to produce 5mls of soluble extract as before. 1ml was retained for the standard incubation while the remainder was centrifuged at 100,000g for 1 hour and the resulting pellet (particulate fraction) resuspended in 0.6ml of buffer. 0.5ml being used in the incubation.

Substrate:

1uCi of $^{3}H$ androstenedione was substituted for the 2.5uCi previously used. Appropriate adjustments were made to the amount of "cold" substrate being added in order to maintain the overall concentration of androstenedione at 100nm.

Reaction time:

The reaction was allowed to proceed overnight (17hrs).

Results:

The results are shown in Table 2:4.

Comment:

The levels of counts now being observed in the assay are markedly reduced in comparison to the original experiments. However, the fact that significant amounts of tritium are being released at the start of the reaction suggests that non-specific release of tritium can still occur readily.

| TIME | BLANK (dpm) | SOLUBLE EXTR. (dpm) | %conv. | PARTICULATE F. (dpm) | %conv. |
|---|---|---|---|---|---|
| 0 | 3402 | 4249 | | 4221 | |
| 17hrs | 5929 | 7956 | 0.1% | 13774 | 0.4% |

TABLE 2:4.

Comparison of a "particulate fraction" with the soluble extract from adipose tissue in the tritium release assay.

Modification 4.

Substitution of 1-beta $^{3}H$ androstenedione for 1-beta,2-beta $^{3}H$ androstenedione as substrate.

A number of reports have suggested that the 2-beta tritium is unstable and is liable to be released non-enzymically leading to high levels of background counts[12,145,164]. The effect of using 1-beta tritiated substrate rather than the 1-beta,2-beta tritiated substrate was therefore studied.

Substrate:

1-beta $^{3}H$ androstenedione was prepared from 1-beta,2-beta $^{3}H$ androstenedione using the method of Berkovitz et. al.[12]. This involves overnight incubation with methanol/0.1 N NaOH (9:1, vol/vol). The steroid was then extracted with methylene chloride and purified by thin layer chromatography in chloroform:acetone (185:15).

Incubations:

2 parallel sets of incubations were performed, each with an albumin "blank", and a particulate preparation of adipose tissue. luCi of 1-beta,2-beta $^{3}H$ androstenedione was used as substrate in the first set and luCi of 1-beta $^{3}H$ androstenedione in the second. Appropriate adjustments were again made to ensure that the overall concentration of androstenedione remained at 100nm in all incubations.

Aliquots (350ul) were withdrawn at two time points (0hrs & 3hrs) and the remainder (400ul) was used for the final (17hr) estimation. Single aliquots of 200ul were counted for each time point.

Results:

The results are shown in Table 2:5.

Comment:

Use of the 1-beta $^{3}H$ androstenedione significantly reduced the counts in both the "blank" and the tissue incubations at all time points. This is consistent with a reduction in the non-specific release of tritium.

| TIME (hrs) | 1ß,2ß $^{3}$H androstenedione | | | 1ß $^{3}$H androstenedione | | |
|---|---|---|---|---|---|---|
| | BLANK (dpm) | PARTICULATE (dpm) | %conv | BLANK (dpm) | PARTICULATE (dpm) | %conv |
| 0 | 1142 | 1269 | | 952 | 1163 | |
| 3 | 2369 | 4781 | 0.13 | 1354 | 2285 | 0.05 |
| 17 | 12375 | 20054 | 0.42 | 3046 | 7002 | 0.22 |

TABLE 2:5.

Comparison of 1ß $^{3}$H androstenedione with 1ß,2ß $^{3}$H androstenedione as substrate in the tritium release assay.

## Validation experiment A.

## Measurement of the loss of tritium which occurs during the tritium release assay.

### Introduction

An important factor which will influence the accuracy of the tritium release assay is any loss of tritiated water which occurs during the procedure. Such losses have to be quantified before valid comparison with the product identification assay is possible.

### Aim:

To quantify the loss of tritiated water that occurs during the tritium release assay.

### Materials & methods:

Tritium labeled water/aqueous products was obtained by incubating luCi of 1-beta, 2-beta androstenedione with placental microsomes and an NADPH-generating system, followed by extraction with chloroform and activated charcoal. 150ul aliquots were dispensed into distilled water (850ul) and either counted (n=3) or subjected to standard incubation conditions (shaken for 3 hours at 37.C) followed by chloroform and charcoal extraction (n=3). Total counts in the "incubated" samples were then compared with counts in the non-incubated samples to determine the loss of tritium occuring during the assay.

### Results:

Total cpm

| Non-incubated samples | Incubated samples |
|---|---|
| 1. 83027 | 1. 65473 |
| 2. 84640 | 2. 70873 |
| 3. 82325 | 3. 66148 |
| Mean 83330 | 67498 |

$$\text{Average recovery of tritium} = \frac{67498}{83330} \times 100\ \%$$

$$= 81\%$$

Conclusion: 19% of tritium is lost during the assay procedure. Results should therefore be adjusted to correct for this.

## Validation experiment B.

### Variation of substrate concentration.

#### Introduction.

Investigation of the aromatase enzyme(s) by others[2,12,29,53] reveals classical Michaelis-Menton kinetics in that the enzyme can be saturated by increasing concentrations of substrate. The concentration of androstenedione in breast adipose tissue is reported to vary between 10 and 200nm[37,46]. The concentration of substrate (100nm) used in the standard assay was therefore chosen to reflect physiological substrate availability.

#### Aim:

To investigate the enzyme kinetics of the tritium release assay to determine if physiological substrate concentrations are appropriate for this assay system.

#### Method:

A particulate fraction was prepared from 4 grams of adipose tissue. Four parallel incubations were then performed with the standard concentrations of cofactors, but with the following concentrations of androstenedione: 33nM, 75nM, 150nM, & 200nM (achieved by varying the amount of radioinert androstenedione added to each incubation. Each incubation still contained 1uCi of 1-beta $^{3}H$ androstenedione)

#### Results:

The assay results are presented in Table 2:6 and Figure 2:3 . The

| Substrate Conc. (nm) | 33 | 75 | 150 | 200 |
|---|---|---|---|---|
| % conversion (3 hrs) | 0.16 | 0.12 | 0.10 | .083 |
| fmol E /mg prot /hr | 18 | 30 | 50 | 54.8 |

Table 2:6.

The effect of increasing substrate concentration on the aromatase assay.

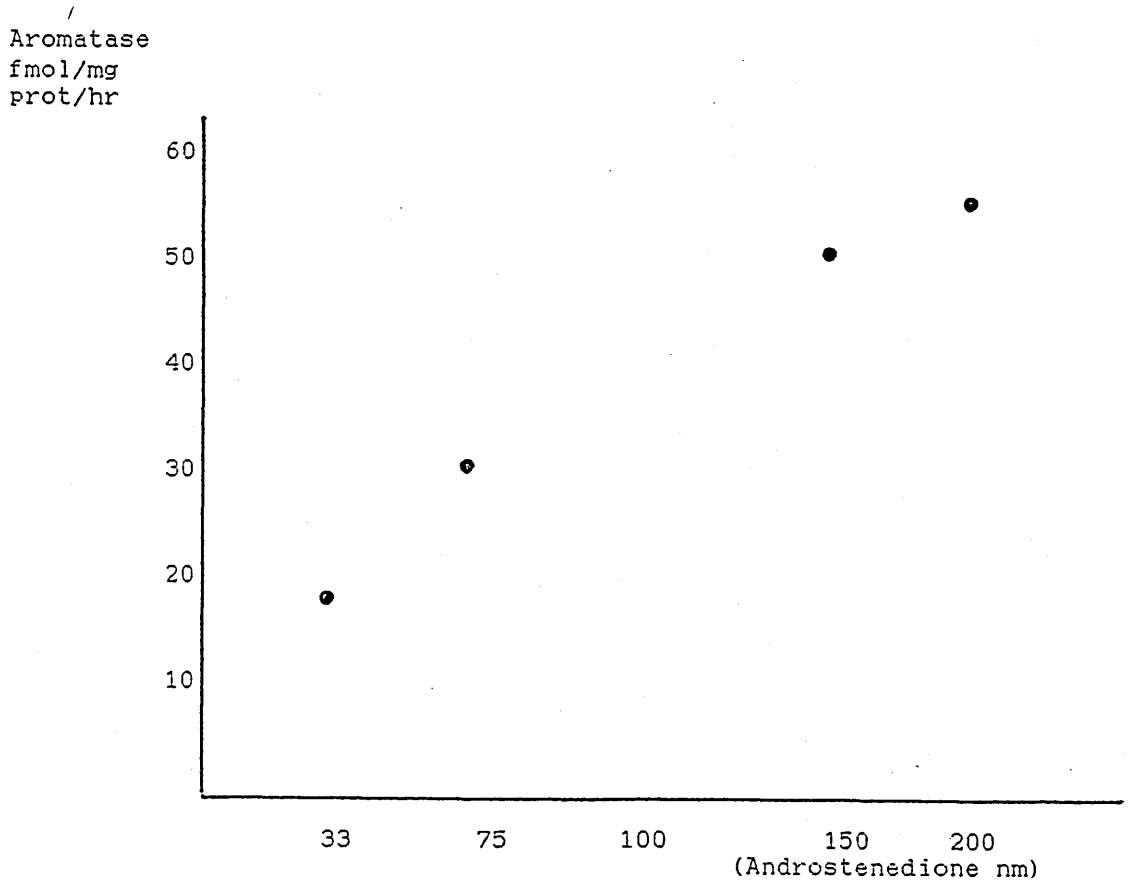



Figure 2:3

The effect of increasing substrate concentration on aromatase activity in adipose tissue.

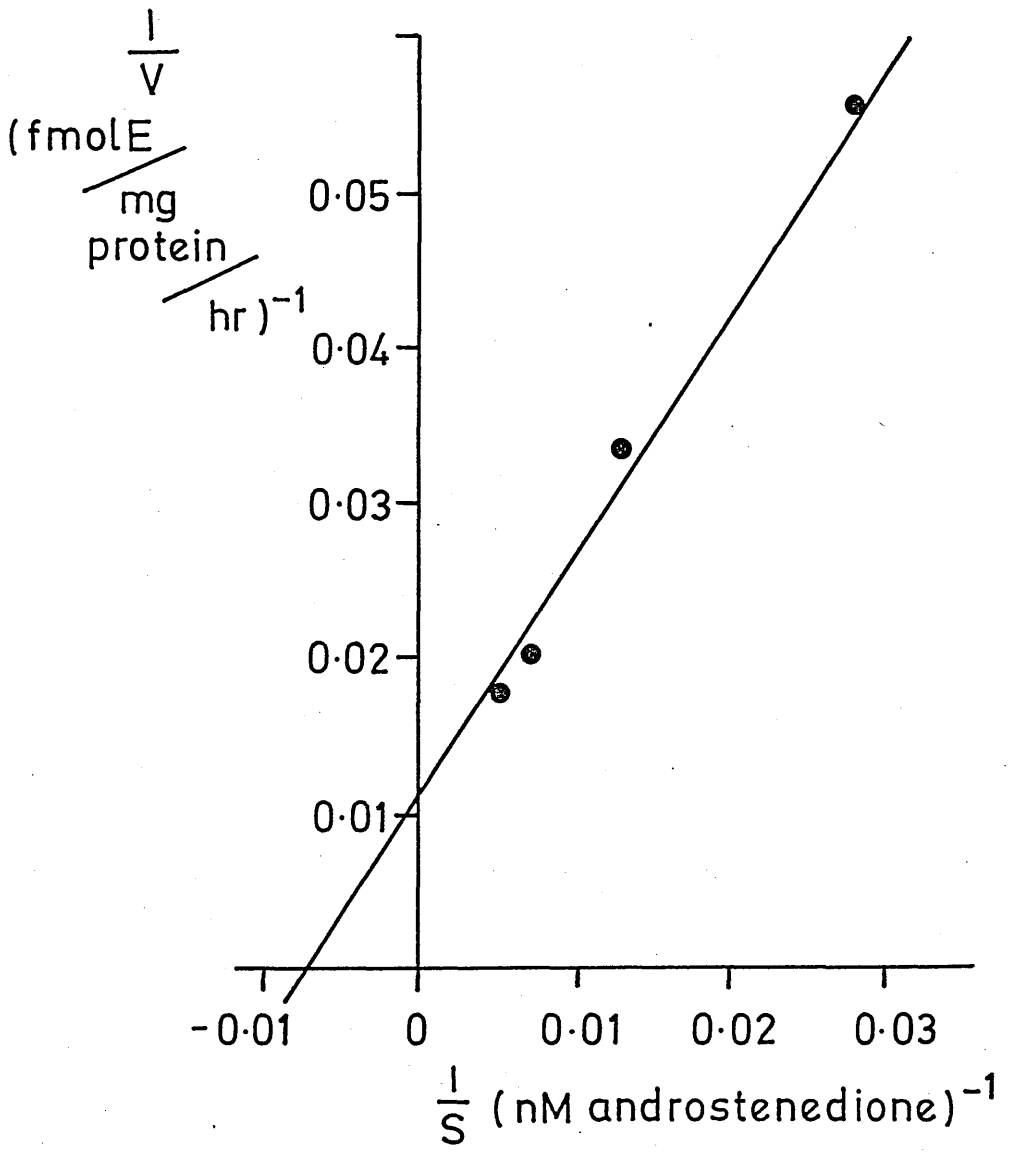



Figure 2:4

Lineweaver-Burke plot of aromatase activity in adipose tissue.

Lineweaver-Burke plot of the results is shown in Figure 2:4 . (correlation coefficient = 0.996, Km = 132nm, Vmax = 89 fmol/mg prot/hr.)

Comment:

These results confirm that in this assay, the reaction follows classical Michaelis-Menton kinetics. Physiological concentrations of androstenedione (10-200nM) do not produce enzyme saturation. The results from the present assay should therefore reflect physiological activity rather than maximal enzyme activity.

Modified tritium release assay.

With incorporation of the foregoing modifications into the "tritium release" assay, the assay procedure has now been finalised and is outlined below and summarised in Figure 2:5.

Aromatase Assay (Tritium release)

Cofactors [nicotinamide (10mM), MgCl2 (5mM), glucose 6-phosphate (10mM), glucose 6-phosphate dehydrogenase (2u/ml) and 2 mM each of NAD, NADP and ATP] and substrate [ 100 nM 1-beta $^{3}H$ androstenedione (1 uCi)] were pre-incubated at 37$^{o}$C in a total volume of 600 ul phosphate buffer (0.1M, pH 7.4). Enzyme reactions were started by addition of the particulate fraction (500 ul) to substrate and co-factors. Blank incubations were also set up using 500 ul of bovine serum albumin (2.0 mg/ml) in place of the particulate fraction. Incubations were then performed overnight at 37$^{o}$C with continuous shaking. An aliquots (550 ul) of the reaction mixture was removed into 3 ml of ice-cold chloroform after 3 hours, the remainder being left to incubate overnight and the reaction terminated by addition of a further 3mls of ice cold chloroform. The aliquots + chloroform were thoroughly shaken, and centrifuged at 2000g for 3 minutes to terminate the reaction. An aliquot (400 ul) of the resulting aqueous fraction was added to 5% charcoal solution (800 ul), mixed, and allowed to stand for 10 minutes with occasional further mixing. The charcoal was then precipitated by centrifugation at 2,000 g for 15 minutes and the supernatant decanted into a glass counting vial containing NE 260 Scintillation fluid (10 ml). Radioactivity was measured on

2 grams of adipose tissue homogenised in phosphate buffer (2ml).

↓

centrifuge (800g 5 minutes)

↓

supernatant centrifuged (100,000g, 1 hour)

↓

pellet (particulate fraction) resuspended in buffer (600 ul).

↓

Cofactors + 1-beta $^{3}H$ androstenedione (600ul) pre-incubated 37$^{o}$C

↓

Particulate fraction or Blank (500ul) added to substrate + co-factors.

↓

Incubate overnight (37$^{o}$C) with continuous shaking.

↓

Aliquot (550 ul) into 3 ml chloroform at 3 and 18 hours.

↓

Aliquots + chloroform shaken + centrifuged (2000g 3mins).

↓

Aliquot (400 ul) of aqueous fraction + 5% charcoal solution (800ul), mixed

↓

Centrifuge (2,000g, 15 mins).

↓

Count $^{3}H$ in supernatant (NE 260 Scintillation fluid 10 ml).

↓

$^{3}H$ in incubation - $^{3}H$ in blank = $^{3}H$ released.

↓

Correct for procedural losses, counting efficiency.

Figure 2:5.

TRITIUM RELEASE ASSAY

a Packard Tri-Carb Liquid Scintillation spectrometer. Counts obtained in the blank incubations were subtracted from all other counts before correction for procedural losses and counting efficiency.

## Validation experiment C.

## Comparison of the modified tritium release assay with the product identification assay.

### Materials & methods:

A series of three parallel incubations were performed on three separate occasions using adipose tissue samples from successive breast cancer patients. On the first occasion product identification assays were performed for 3 hours and overnight (18 hours). An aliquot from the tritium release assay being taken at 3 hours and the remainder allowed to incubate overnight. For the next two comparisons all incubations were performed for 3 hours.

### Results:

The results are shown in Table 2:7.

### Discussion:

In the 3 hour incubations the tritium release assay has detected 68%, 70% and 67% of the activity measured by the product identification assay. These figures are consistent with the findings of Thomson & Siiteri[156] and Ackerman et al[2] who have noted that only 70% of the tritium in the 1¢ position is released when oestrogen is formed. There is no correlation however, between the assays for the overnight incubations. This presumably reflects further metabolism of oestrogens in the overnight incubations (since less radioactively labeled oestrogen is present after 20 hours than at 3 hours). The validity of overnight incubations is therefore difficult to verify so it was decided to

| Incubation | %conversion "tritium release" | %conversion "oestrogen extraction" |
|---|---|---|
| 1a 3hrs | 0.052 | 0.076 |
| 1b 18hrs | 0.302 | 0.050 |
| 2 3hrs | 0.037 | 0.053 |
| 3 3hrs | 0.048 | 0.072 |

Table 2:7.

Comparison of the tritium release assay with the product identification assay.

proceed only with 3 hour incubations.

If this correlation is consistently reproducible, then it appears that the tritium release assay can accurately measure oestrogen formation in adipose tissue.

## Validation experiment D.

Aim.

a) Further comparison of the tritium release and product identification assays.

b) Verification that the assays are measuring activity of the aromatase enzyme(s) by incorporating aromatase inhibitors (aminoglutethimide and 4-OH androstenedione) in both assay systems.

Materials & methods:

As before, apart from: 20g of adipose tissue (obtained from patient 10) was used to produce 5ml of particulate fractions. 3 pairs of parallel incubations were performed by the two methods - a) No inhibitor, b) + 0.1mM aminoglutethimide, c) + 0.1uM 4-OH androstenedione (such concentrations are known to inhibit aromatase activity in assay systems utilising extracts of placenta, ovary and breast tumours[21,106,110,120,167]).

Results:

See Table 2:8.

Comment:

Again there is a consistent relationship between the results of the two different assays with the tritium release assay recording 73%, 66%, and 75% of the activity detected in the product identification assays. In addition, there has been about 50% inhibition of activity by aminoglutethimide and 40% inhibition by 4-OH Androstenedione. While such inhibition is not as marked as that obtained in other tissues (placenta, ovary,

| Incubation | %conversion "tritium release" | %conversion "oestrogen extraction" |
|---|---|---|
| A (no inhibitor) | 0.048 | 0.066 |
| B (+ 0.1mM AMG) | 0.023 | 0.035 |
| C (+ 0.1uM 4OHA) | 0.028 | 0.037 |

Table 2:8.

Effect of aromatase inhibitors on the tritium release and product identification assays.

tumour[21,106,110,120,167]) it is important to have established this effect since these agents appear to be able to reduce peripheral aromatase in vivo[21].

In six comparisons which have been made between the assays for 3 hour incubations the tritium release assay has detected activity which is between 66 and 75% of that measured by the product identification assay. The average discrepancy between the assays is 70% and is thus in keeping with the observations of others[2,156] that only 70% of the tritium is released from 1-beta $H^3$ androstenedione when oestrogen is formed.

If the tritium release results are adjusted to correct for this discrepancy (by dividing the results by 0.70) the comparison of the two assays is as shown in Table 2:9 and Figure 2:6, revealing an excellent correlation (correlation coefficient = 0.988 $p < 0.001$).

The tritium release assay in its present form therefore appears to be a suitable assay for the measurement of aromatase activity in adipose tissue.

| Incubation | %conversion "tritium release" | %conversion "oestrogen extraction" |
|---|---|---|
| 1a | 0.074 | 0.076 |
| 2 | 0.053 | 0.053 |
| 3 | 0.066 | 0.072 |
| A | 0.069 | 0.066 |
| B | 0.032 | 0.035 |
| C | 0.040 | 0.037 |

Table 2:9.

Comparison of the tritium release assay with the oestrogen extraction assay.

(The tritium release results have been divided by 0.7 to allow for incomplete release of tritium)

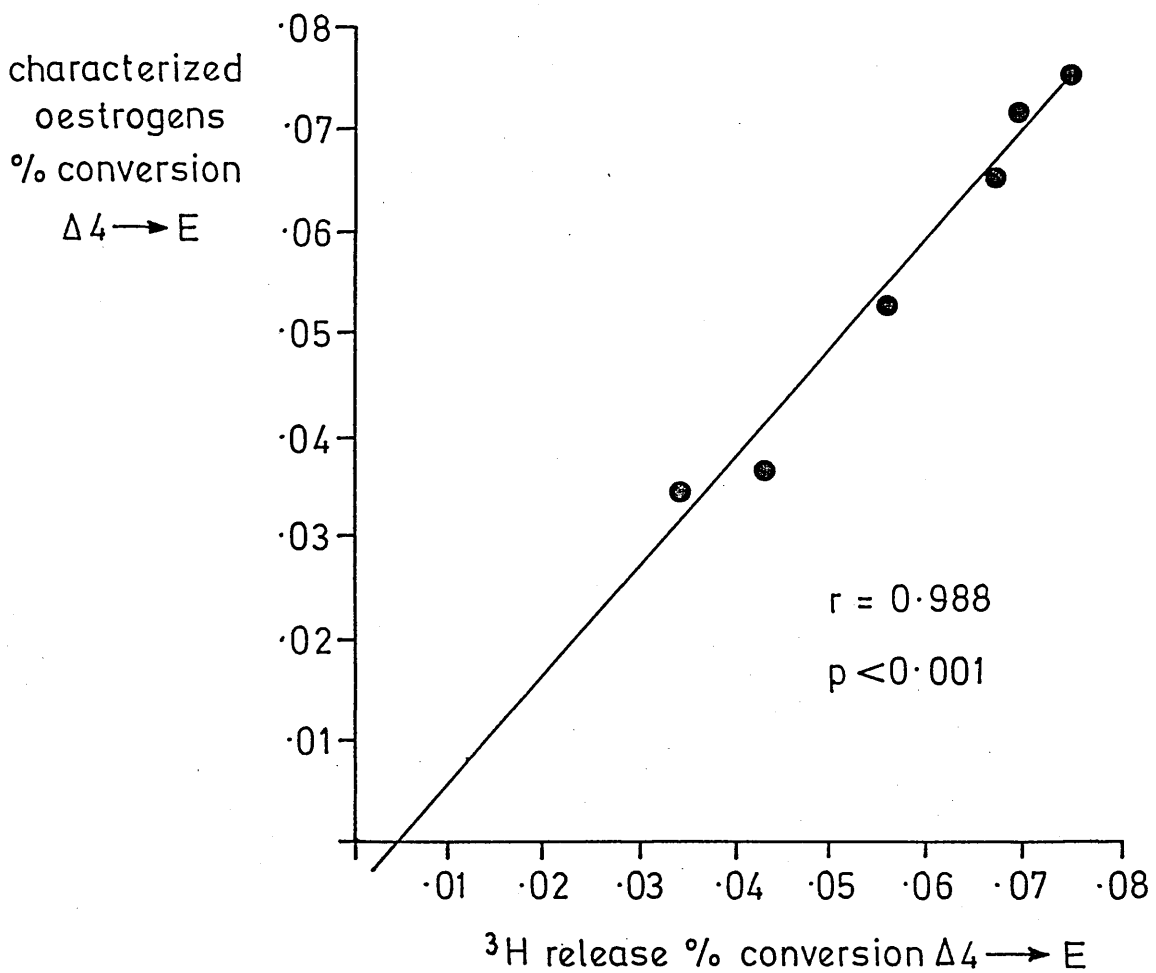



Figure 2:6

Comparison of the product extraction and tritium release assays for aromatase activity in adipose tissue.

## Validation experiment E.

Aim:

To investigate the relationship between the quantity of adipose tissue assayed, the protein concentration of particulate fractions and aromatase activity.

Method:

Six grams of breast adipose tissue was obtained from one patient and homogenised to produce 6 mls of soluble extract. This was divided into aliquots of 1, 2, & 3 mls from which particulate fractions were prepared, each being resuspended in 600ul of buffer. 500ul of this suspension was incubated in the standard tritium release assay and the remainder retained for protein estimation.

Protein estimation:

The protein-dye binding method of Bradford[17] was utilised. Aliquots (50ul) of the resuspended particulate fractions were added to 0.9% NaCl (250ul). Duplicate aliquots (100ul) were then thoroughly mixed with 5ml of an acidified solution of Coomassie Brilliant Blue G-250 [100mg dissolved in 95% ethanol (50ml), 85% phosphoric acid (100ml) diluted to a final volume of 1 liter]. Absorption was measured at 595nm on a Unicam SP600 spectrophotometer against a reagent blank.

Results: The results are outlined in Table 2:10.

Comment: It is clear from these results that aromatase activity in a particulate fraction of adipose tissue is directly related to the protein concentration of the tissue extracts which is in turn related to the quantity of tissue included in the assay.

| Millitres of soluble extract (equivalent to grams tissue) | 1 | 2 | 3 |
|---|---|---|---|
| Protein concentration(mg/ml) of particulate fraction | 2.0 | 3.8 | 5.0 |
| % convertion at 3 hours | 0.022 | 0.044 | 0.054 |
| Aromatase activity (fmol/mg prot./hr) | 22 | 24 | 21 |

Table 2:10.

The effect of increasing quantity of tissue on the protein concentration of the particulate fraction and its aromatase activity.

## Validation experiment F.

## The effect of tissue storage.

### Aim:

To determine the effect on assay results of storing adipose tissue at $-20^{o}C$ and $-196^{o}C$.

### Introduction

The study of a large series of tissue samples is facilitated if samples can be stored after collection without significant loss of enzyme activity. The purpose of this experiment was to investigate the effect of tissue storage on assay results.

### Materials & methods:

Four grams of tissue was obtained from each of nine patients. After separation of adipose tissue from parenchyma and fibrous tissue two grams was assayed immediately and the remaining two grams stored in liquid nitrogen ($-196^{o}C$ n=7) or in the deep freeze ($-20^{o}C$ n=2). Storage time varied from 2 days to 2 months (Table 2:11) before tissue was removed thawed and assayed. Ten grams of tissue was obtained from a further case. Two grams was assayed immediately and the remainder stored in 2 gram aliquots ($-196^{o}C$) for assay after various storage times. All assays were performed by the tritium release method outlined previously. At the time of assay aliquots were taken from all samples for protein estimation.

| Patient No. | 3 | 36 | 43 | 51 | 53 | 37 | 13 | 28 | 20 |
|---|---|---|---|---|---|---|---|---|---|
| Temperature (°C) | -20 | -20 | -196 | -196 | -196 | -196 | -196 | -196 | -196 |
| Duration (days) | 60 | 20 | 15 | 4 | 4 | 4 | 2 | 2 | 2 |

Table 2:11.

Tissue storage temperatures and duration.

Results.

The results of the nine paired assays are shown in Table 2:12, and the multiple assay results from patient No 42 in Table 2:13.

Conclusion.

The results indicate that there is negligible loss of enzyme activity with storage of tissue at these temperatures for up to three months. The results again highlight the importance of the protein concentrations of the tissue extracts in relation to enzyme activity. Although in the majority of cases only small variations in protein concentration occured, occasional larger differences are seen - with quite a marked effect on assay results. Such differences could be due to the heterogeneity of the tissue being sampled or to variable tissue processing. Whatever the cause of the variation, assay results were more reproducible if expressed in terms of the amount of protein present. Since levels of protein are likely to be a reflection of the cellularity of the tissue, this will to some extent correct for variations in cellularity between tissue samples.

Table 2:12.

Effect of tissue storage on aromatase assay.

| Patient No. | Initial assays | | | After storage | | |
|---|---|---|---|---|---|---|
| | % 3hrs | Prot(mg/ml) | fmol/mg/hr | % 3hrs | Prot(mg/ml) | fmol/mg/hr |
| 3 | 0.090 | 1.20 | 50.0 | 0.088 | 1.20 | 48.8 |
| 36 | 0.074 | 0.60 | 82.2 | 0.080 | 0.6C | 88.8 |
| 43 | 0.015 | 1.50 | 6.7 | 0.011 | 1.12 | 6.5 |
| 51 | 0.093 | 1.50 | 41.3 | 0.176 | 3.10 | 37.8 |
| 53 | 0.105 | 1.60 | 43.8 | 0.104 | 1.50 | 46.2 |
| 37 | 0.038 | 1.76 | 14.4 | 0.040 | 2.16 | 12.3 |
| 13 | 0.028 | 1.14 | 16.4 | 0.036 | 1.60 | 15.0 |
| 28 | 0.046 | 1.14 | 26.9 | 0.034 | 0.84 | 27.0 |
| 20 | 0.078 | 1.75 | 29.9 | 0.093 | 2.14 | 29.0 |

| Storage Time (days): | 0 | 2 | 15 | 30 | 90 |
|---|---|---|---|---|---|
| Assay Result (%conv) | 0.039 | 0.047 | 0.035 | 0.055 | 0.039 |
| Protein Conc. (mg/ml) | 0.81 | 0.92 | 0.70 | 1.02 | 0.80 |
| Result (fmol/mg prot/hr) | 32.1 | 34.1 | 33.3 | 35.9 | 32.5 |

Table 2:13.

Results of repeated assays after tissue storage at -196°C

## Chapter 3.

# Aromatase activity in breast adipose tissue from women with benign and malignant breast conditions.

Aim:

To compare aromatase activity in breast adipose tissue samples from women with breast cancer and non-malignant breast conditions.

Materials & methods:

Patients:

Adipose tissue was obtained from breast cancer patients at the time of mastectomy or wide local excision and from patients having excision biopsies performed for benign conditions. Prior to surgery, some of the breast cancer patients had been treated by radiotherapy, hormonal therapy and/or chemotherapy. Full details of the patients studied are given in Tables A:1 and A:2 in appendix A. A brief summary of patient details is given in Table 3:1. Patients were considered to be post-menopausal if more than 3 years had elapsed since their last menstrual period. Histological details and results of oestrogen receptor analyses on the associated mastectomies/breast biopsies are given in Tables A:3 and A:4 in appendix A.

Tissues:

At operation adipose tissue close to the site of the breast lesions was removed and transferred to the laboratory on ice. All procedures were then performed at $4^{o}C$ unless otherwise stated. Samples were rinsed in 0.1M phosphate buffer before carefully dissecting adipose tissue free from breast parenchyma and fibrous tissue. Samples were then stored at $-196^{o}C$ until assayed.

TABLE 3:1. Details of the patients studied.

| | BREAST CANCER | BENIGN BREAST DISEASE |
|---|---|---|
| TOTAL | 51 | 36 |
| POST-MENOPAUSAL | 21 | 7 |
| PRE- & PERI-MENOPAUSAL | 30 | 29 |
| MEAN AGE (YEARS) | 53 (31-86) | 45 (29-65) |
| MEAN WEIGHT (KG) | 66 (47-89) | 64 (52-71) |

Assays:

Tritium release assays were performed on particulate fractions from the tissue samples according to the method in Chapter 2. Results are expressed as fmol oestrogen/mg protein/hr (units) on the basis of the conversions at 3 hours.

Statistics:

Statistical analyses were by the Wilcoxon rank test.

Results:

Oestrogen biosynthesis was detected in all samples of breast adipose tissue examined with activities varying from 3.1 to 114 fmol oestrogen produced / mg protein / hour (units). Individual assay results are shown in Tables B:1 and B:2 in Appendix B. The results are compared in Figure 3:1 which shows that the median activity in tissue from breast cancer patients (27.0 units) was more than two-fold higher than that in samples from women with benign conditions (12.3 units). The difference between the groups was significant ($p < 0.0001$).

As measurements were performed in consecutive patients from whom it was possible to obtain sufficient tissue for analysis, the groups have not been matched in any respect and therefore it is important to look for correlations with other factors which might influence oestrogen biosynthesis. The relationship with age of the subjects is plotted separately in Figure 3:2 for patients with either benign or malignant lesions. In neither group of patients was there an obvious relationship between levels of activity and age, although a tendency was apparent for the highest levels of aromatase to be

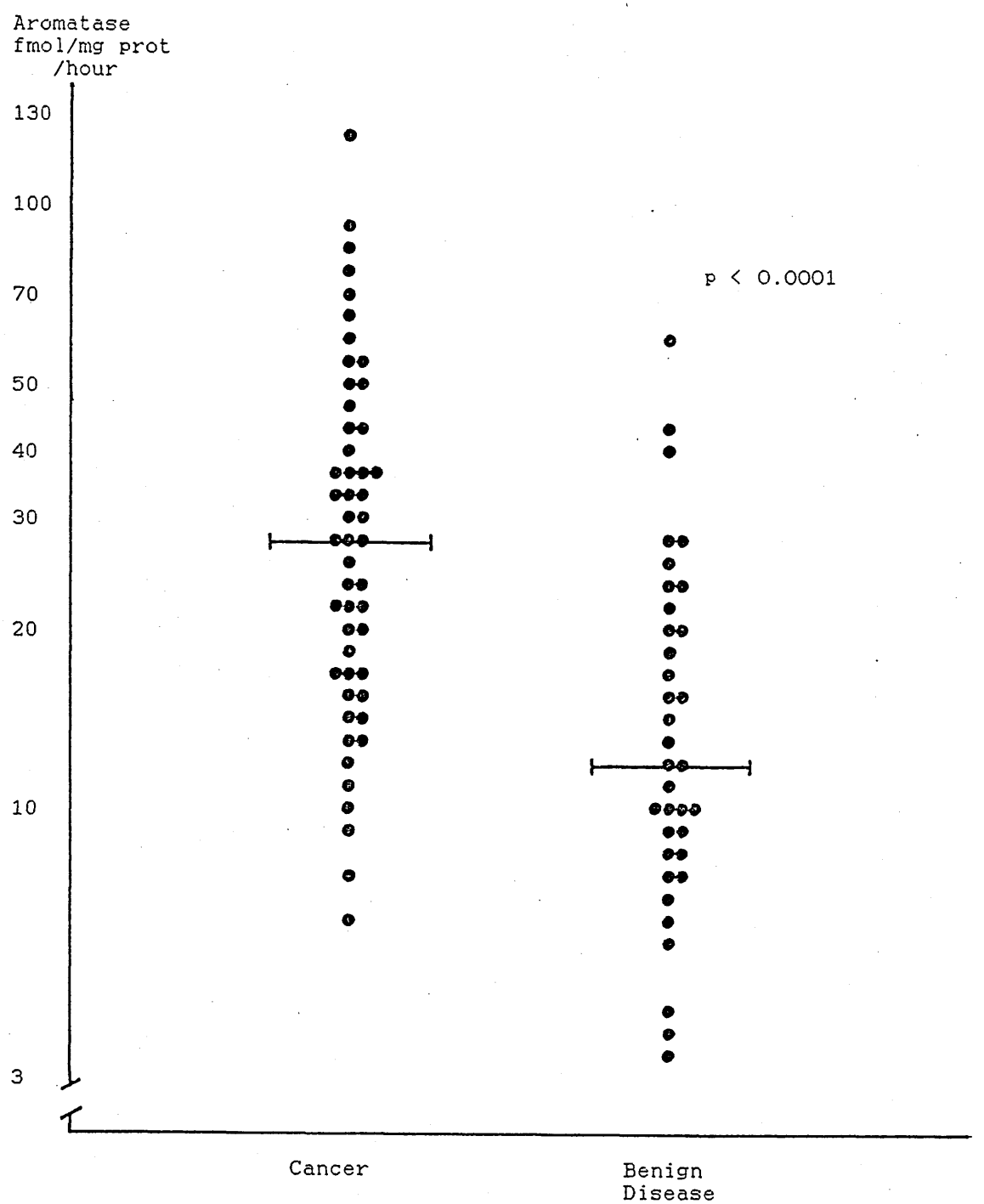



Figure 3:1.

Aromatase activity in breast adipose tissue from women with breast cancer or benign breast disease.

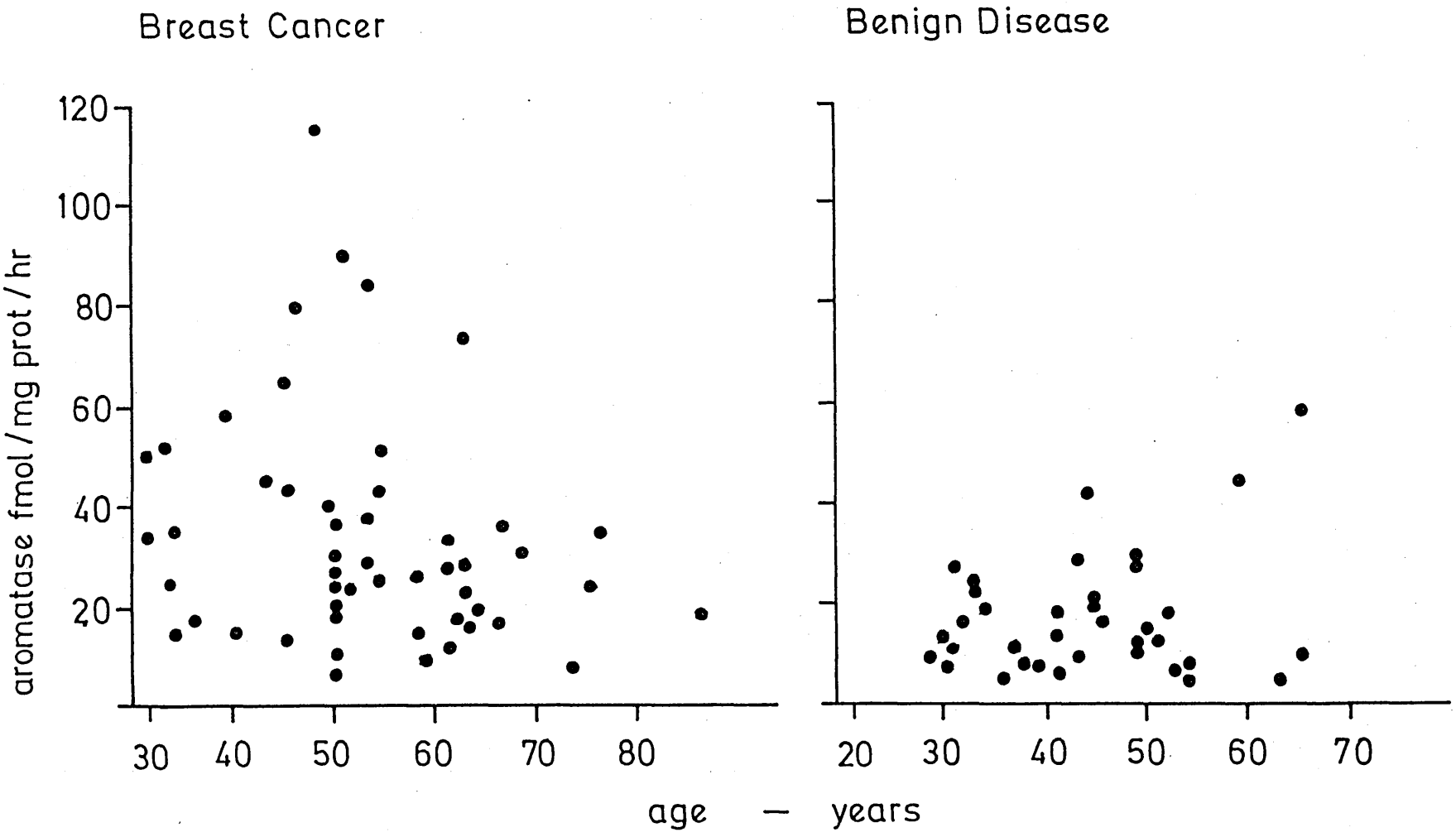



Figure 3:2. Aromatase activity in breast adipose tissue related to age at diagnosis.

found in breast fat from cancer patients between 40 and 55 years of age. The effect of menopausal status on aromatase activity in breast fat from cancer patients is shown in Figure 3:3. The median value (32 units) in pre- and peri-menopausal patients was higher than that in post-menopausal patients (23 units), but the difference was not statistically significant. Meaningful analysis of the benign group was not possible because of the small number of post-menopausal women with non-malignant breast conditions. Since there were marked differences between the cancer and benign groups in terms of both age and menopausal status, it was of interest to compare results using pre- and peri-menopausal patients only, thus minimising the age discrepancy between the groups. As is shown in Figure 3:4, aromatase activity remained significantly higher in the cancer group ($p < 0.0001$).

To determine whether the previous treatment of some of the breast cancer patients could account for the differences seen, levels of aromatase activity in the previously treated group were compared with activities in the group of untreated cancer patients (Figure 3:5). There was no significant diference in levels of activity between the two groups. Additionally, within the group of previously treated cancer patients there was no difference in the levels of aromatase activity between those who responded to systemic therapy compared with those who failed to respond (Figure 3:6). Comparison of aromatase activities between the untreated cancer patients and the benign disease group confirms that significantly higher activity is still present in this group of cancer patients (Figure 3:7).

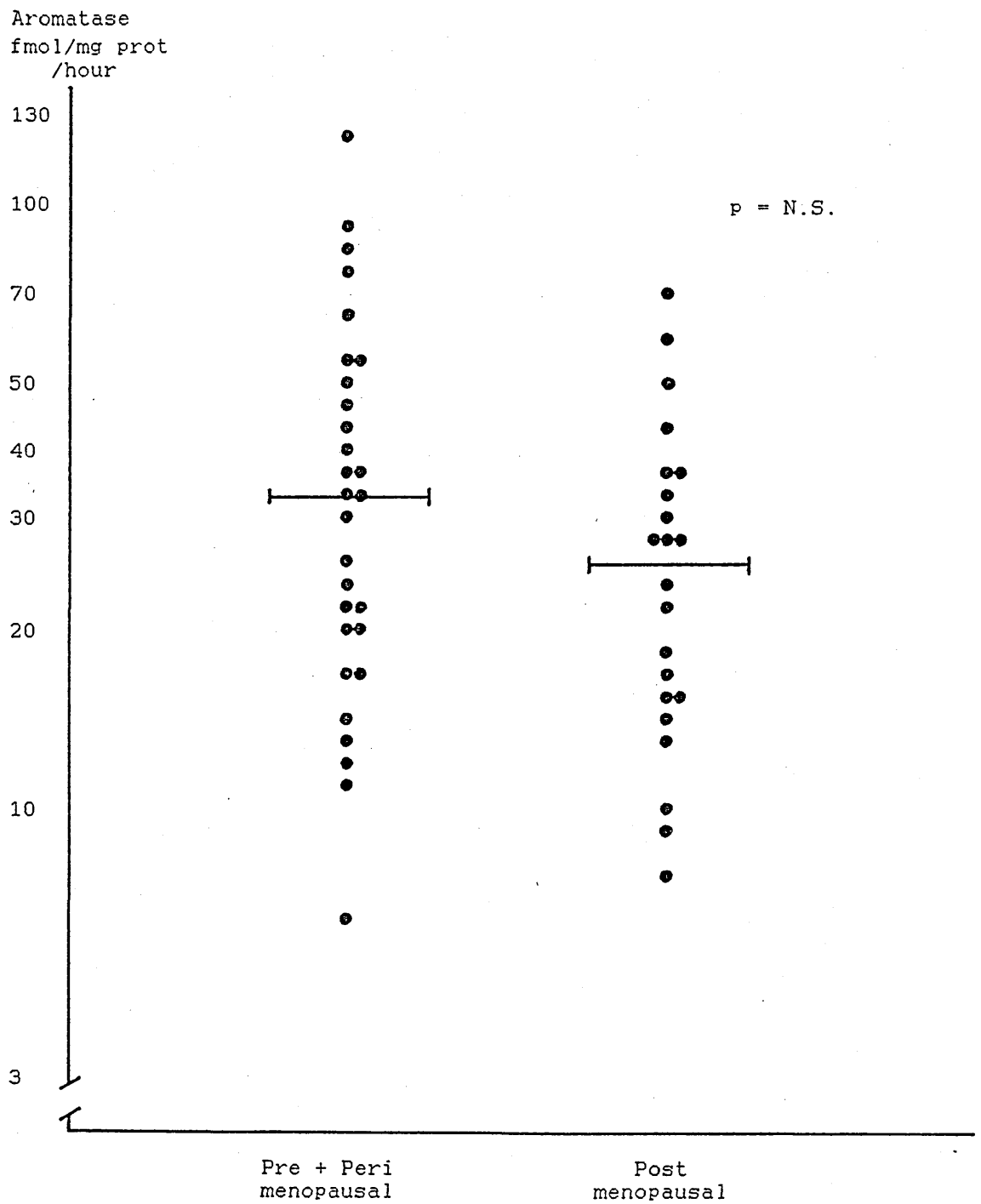



Figure 3:3.

Aromatase activity in breast adipose tissue of breast cancer patients related to menopausal status

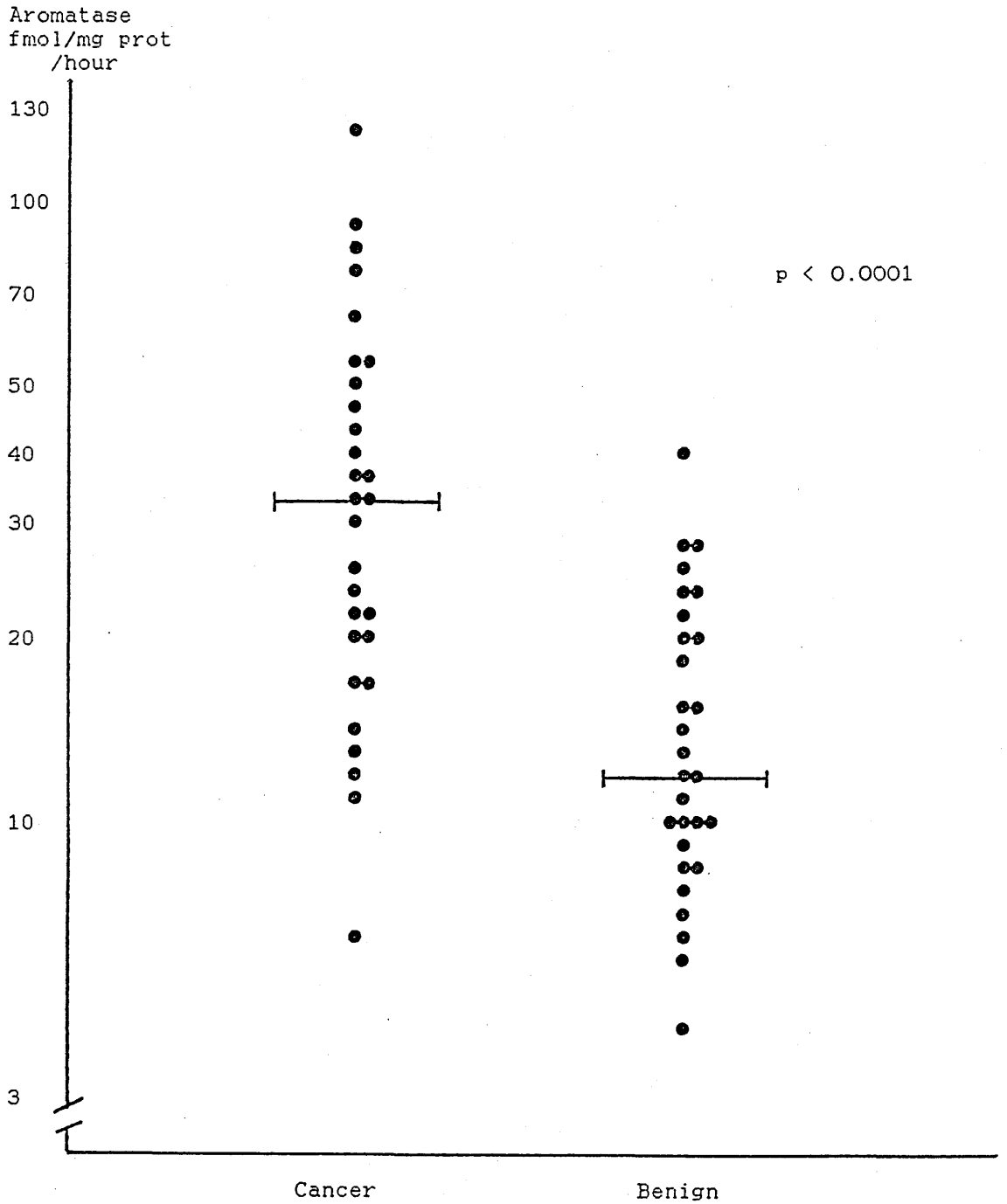



Figure 3:4.

Aromatase activity in breast adipose tissue from pre and peri menopausal women with breast cancer or benign breast disease

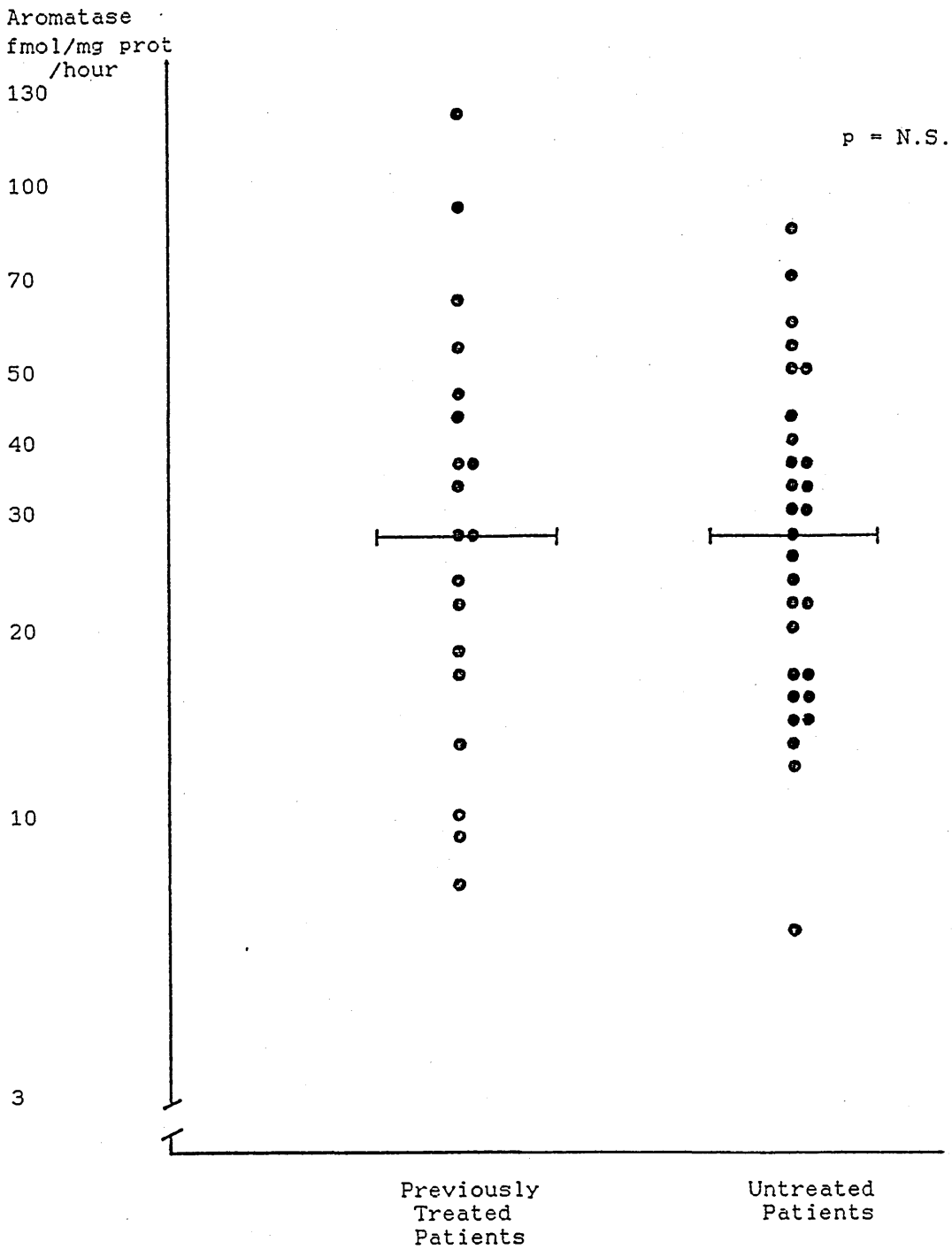



Figure 3:5.

Adipose tissue aromatase activity in treated and untreated breast cancer patients.

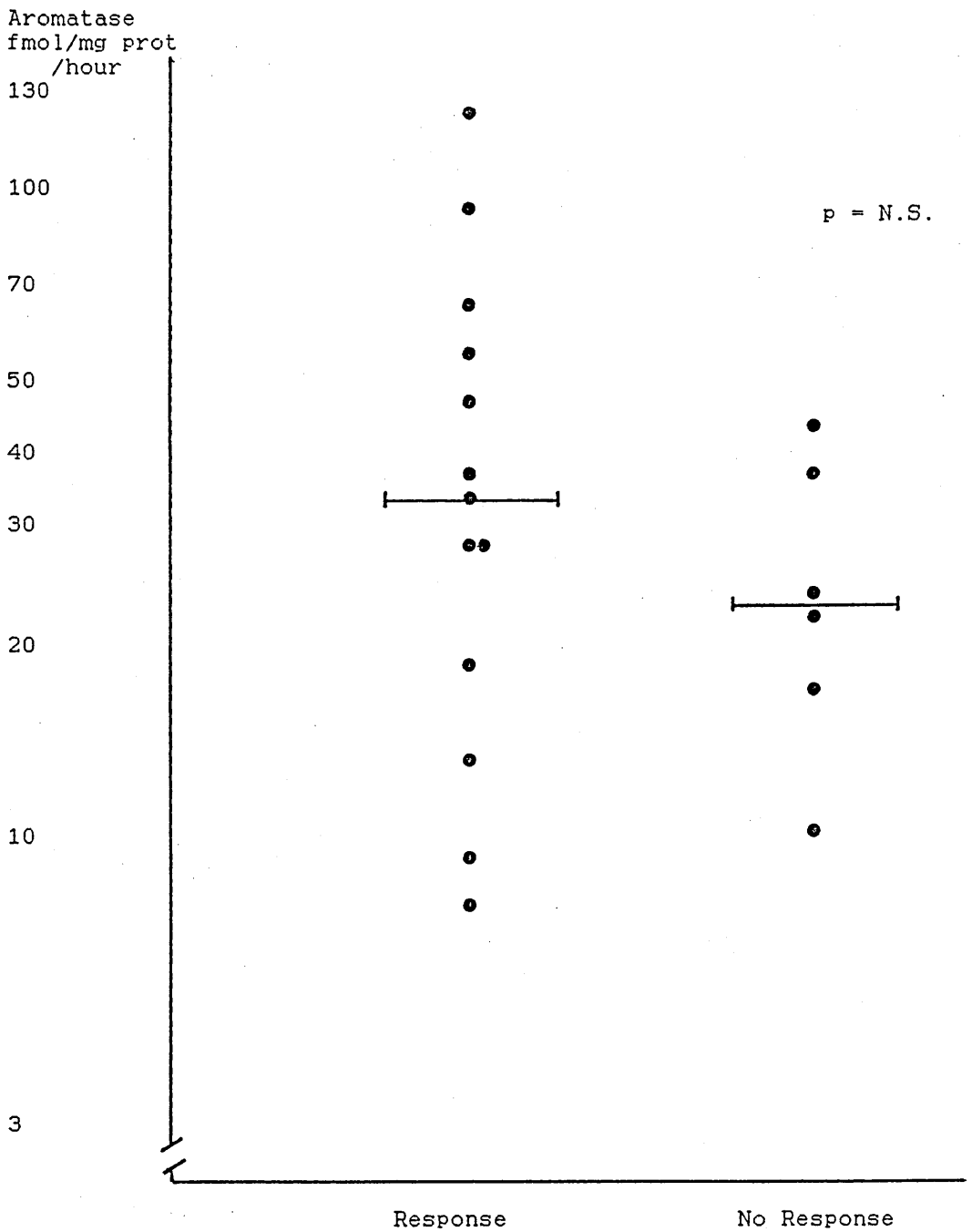



Figure 3:6.

Adipose tissue aromatase activity and response to prior treatment.

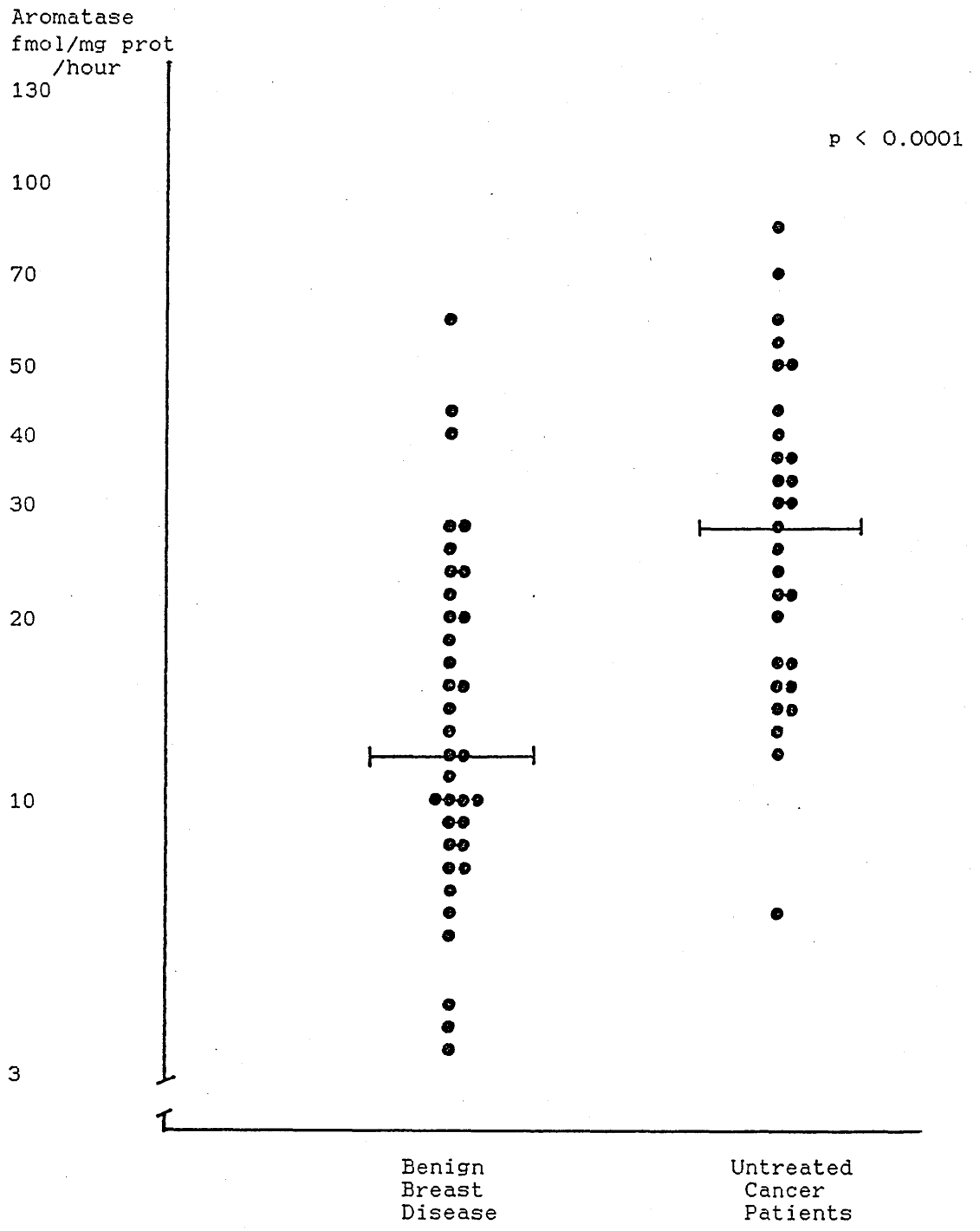



Figure 3:7.

Adipose tissue aromatase activity in untreated breast cancer patients and patients with benign breast disease.

The results were also analysed with regard to height, weight, obesity (Quetlet index, $wt/ht^2$), parity, age at menarche, age at first full term pregnancy, and family history of breast cancer (Figures 3:8-3:14) but no trends were detected. Furthermore, in the cancer group there was no relationship between aromatase activity and tumour type, size or oestrogen receptor protein content. (Figures 3:15-3:17).

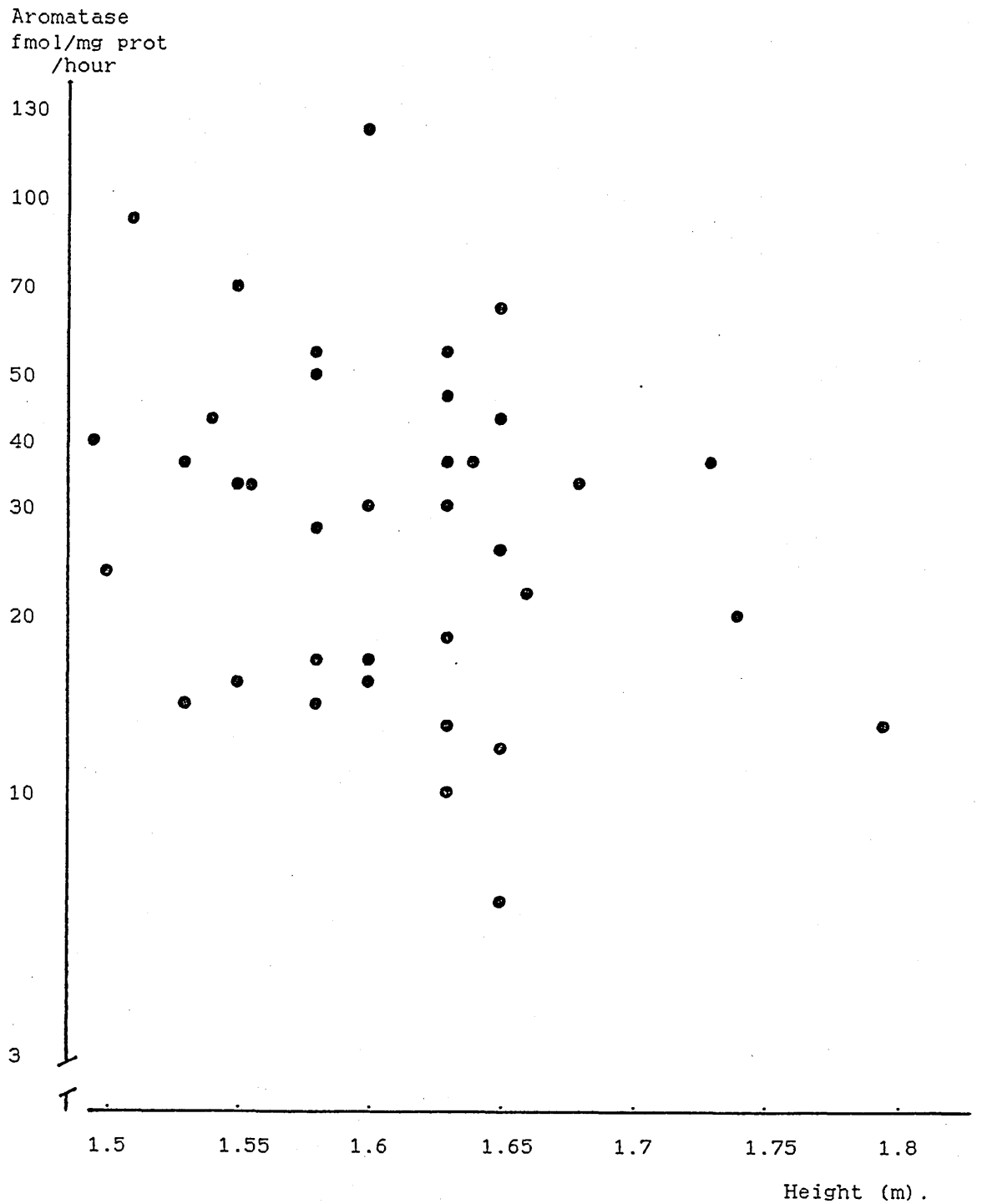



Figure 3:8a.

Adipose tissue aromatase activity and patient height (cancer patients).

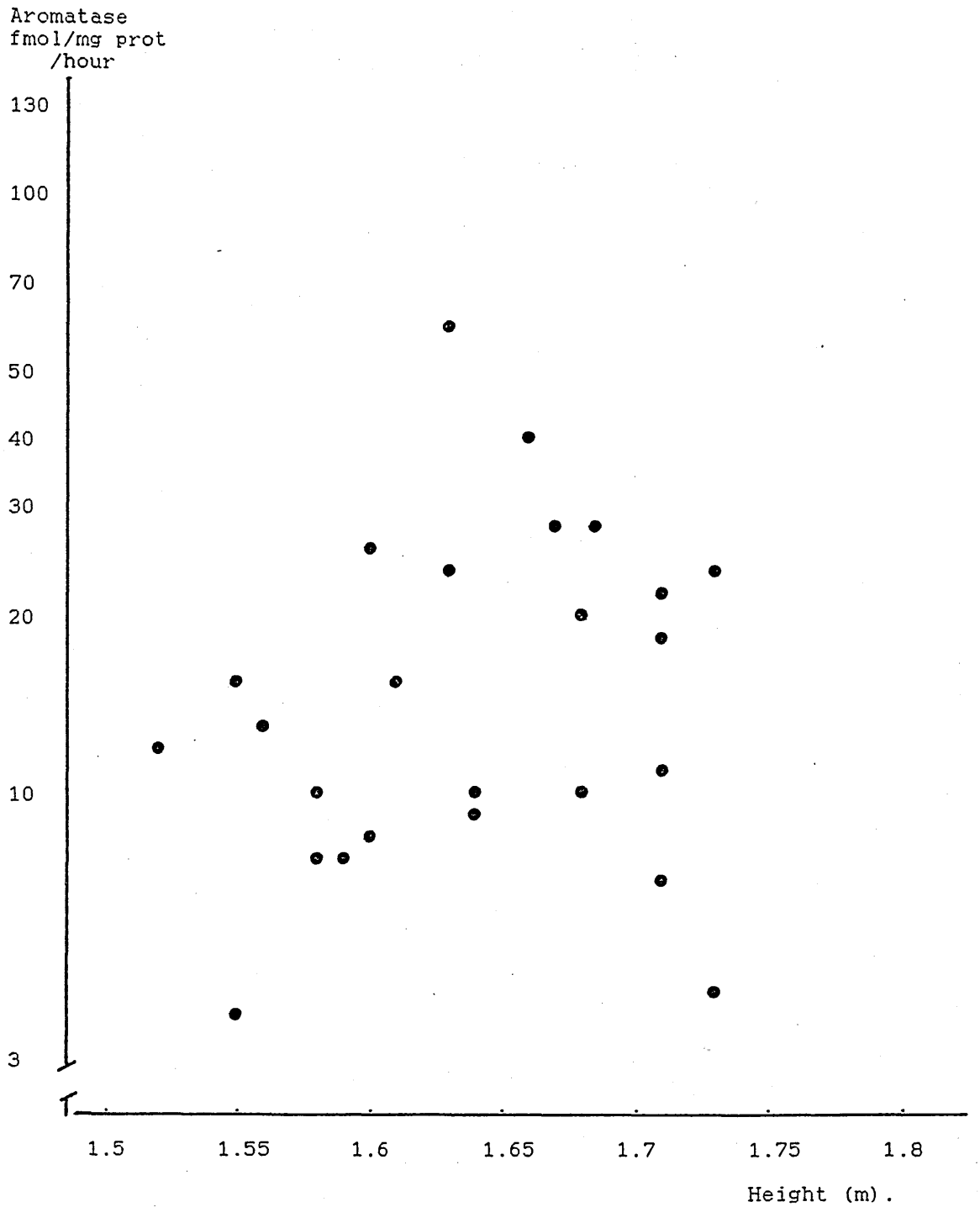



Figure 3:8b.

Adipose tissue aromatase activity and patient height (benign disease).

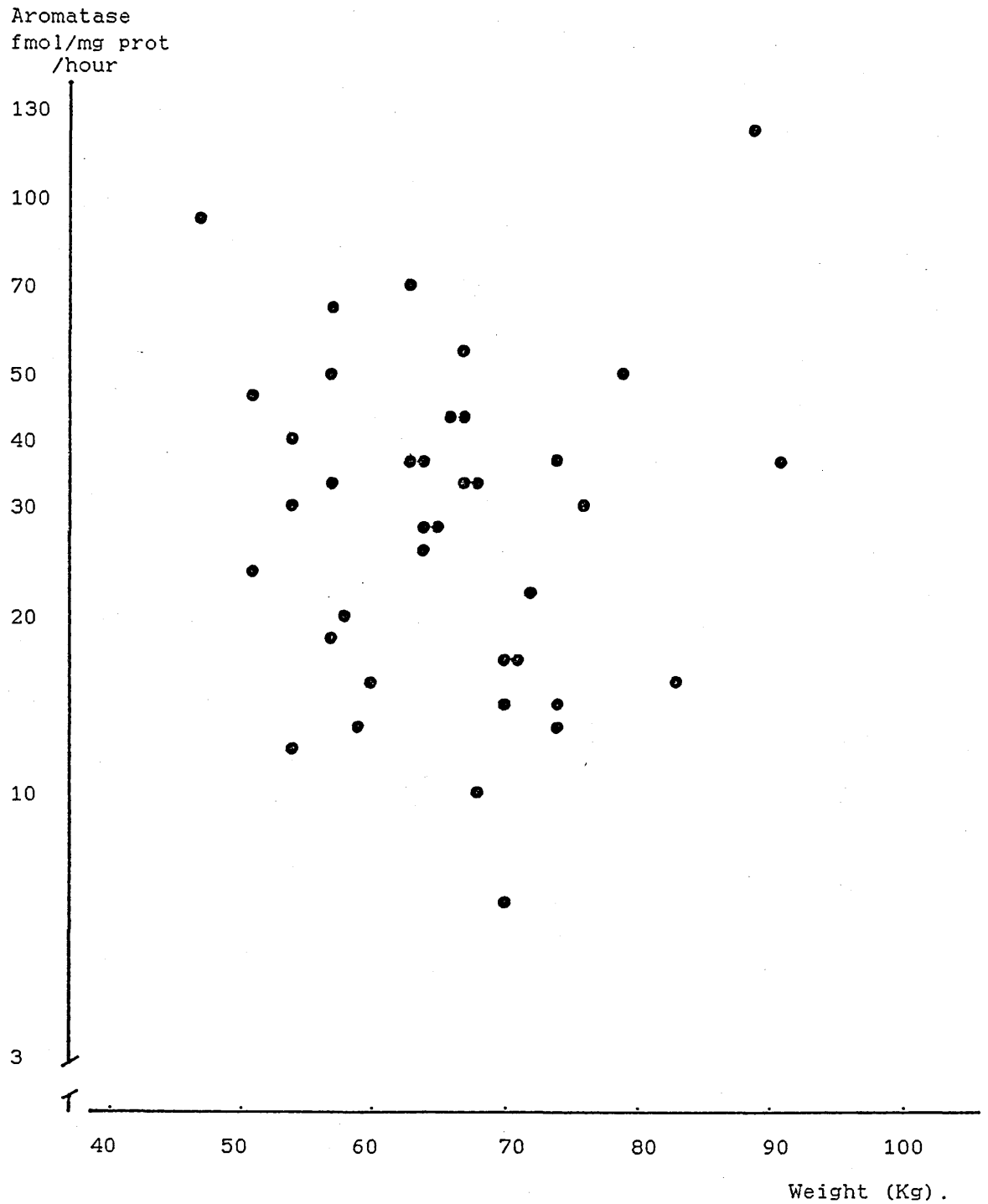



Figure 3:9a.

Adipose tissue aromatase activity and patient weight (cancer patients).

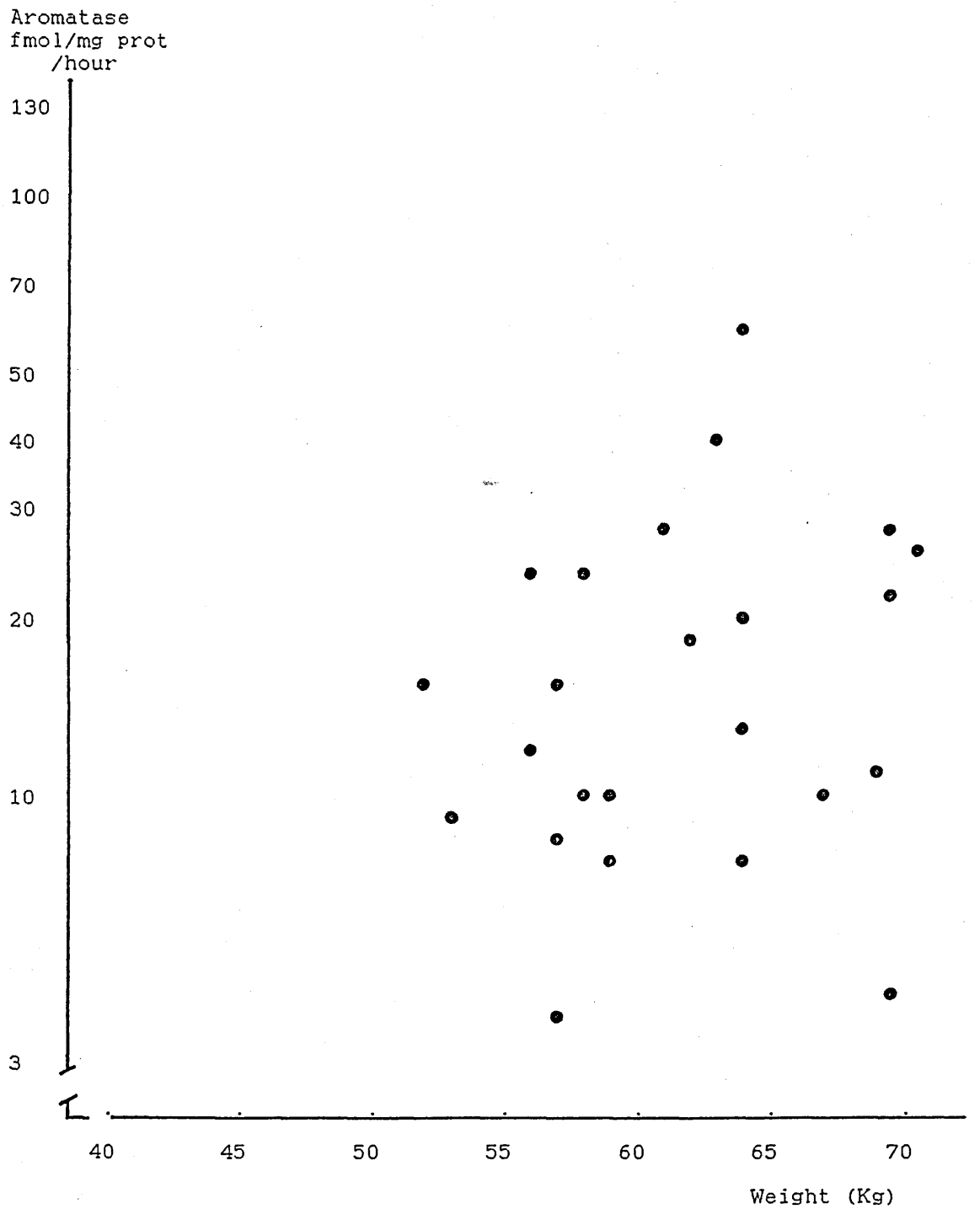



Figure 3:9b.

Adipose tissue aromatase activity and patient weight (benign disease).

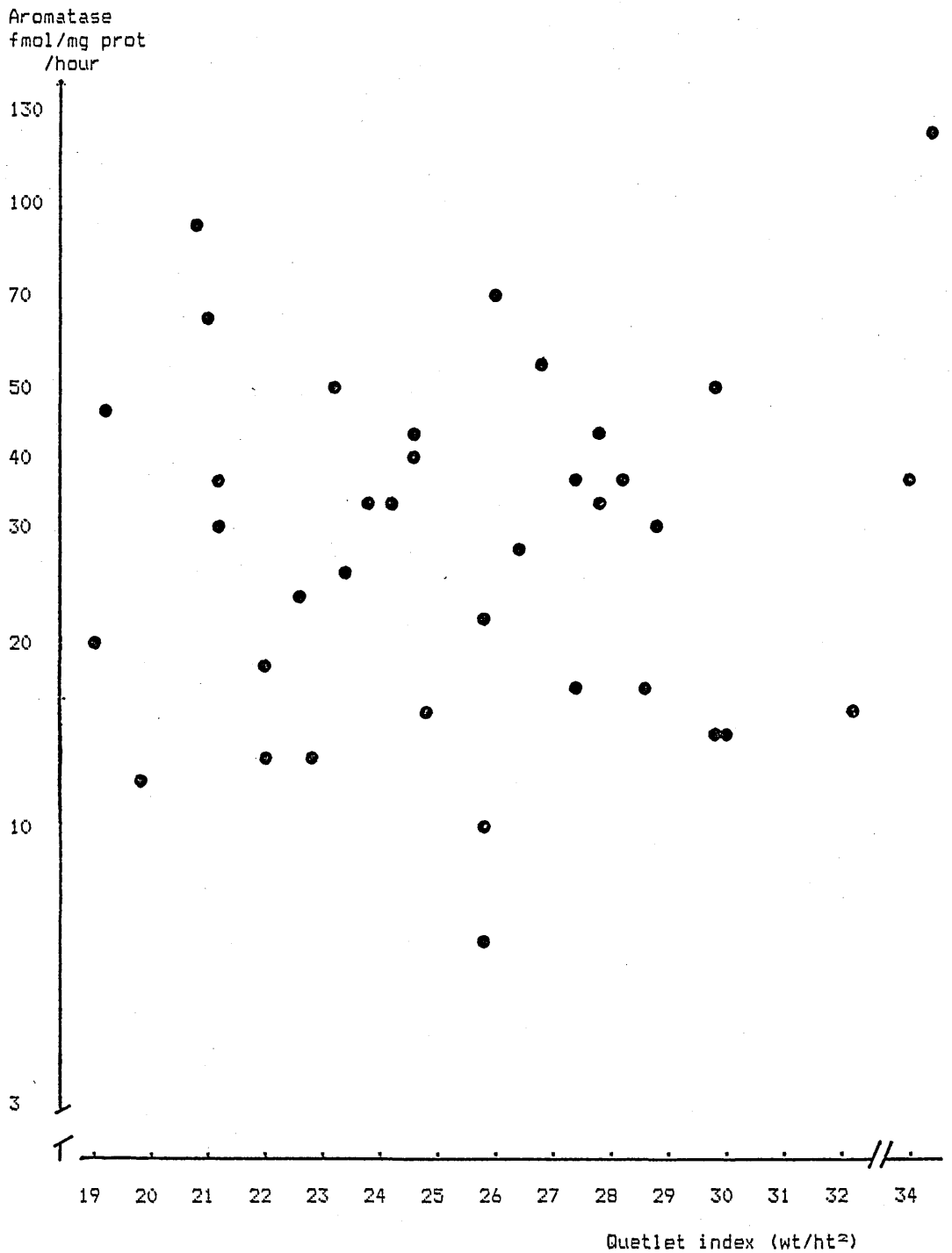



Figure 3:10a.

Adipose tissue aromatase activity and obesity (cancer patients).

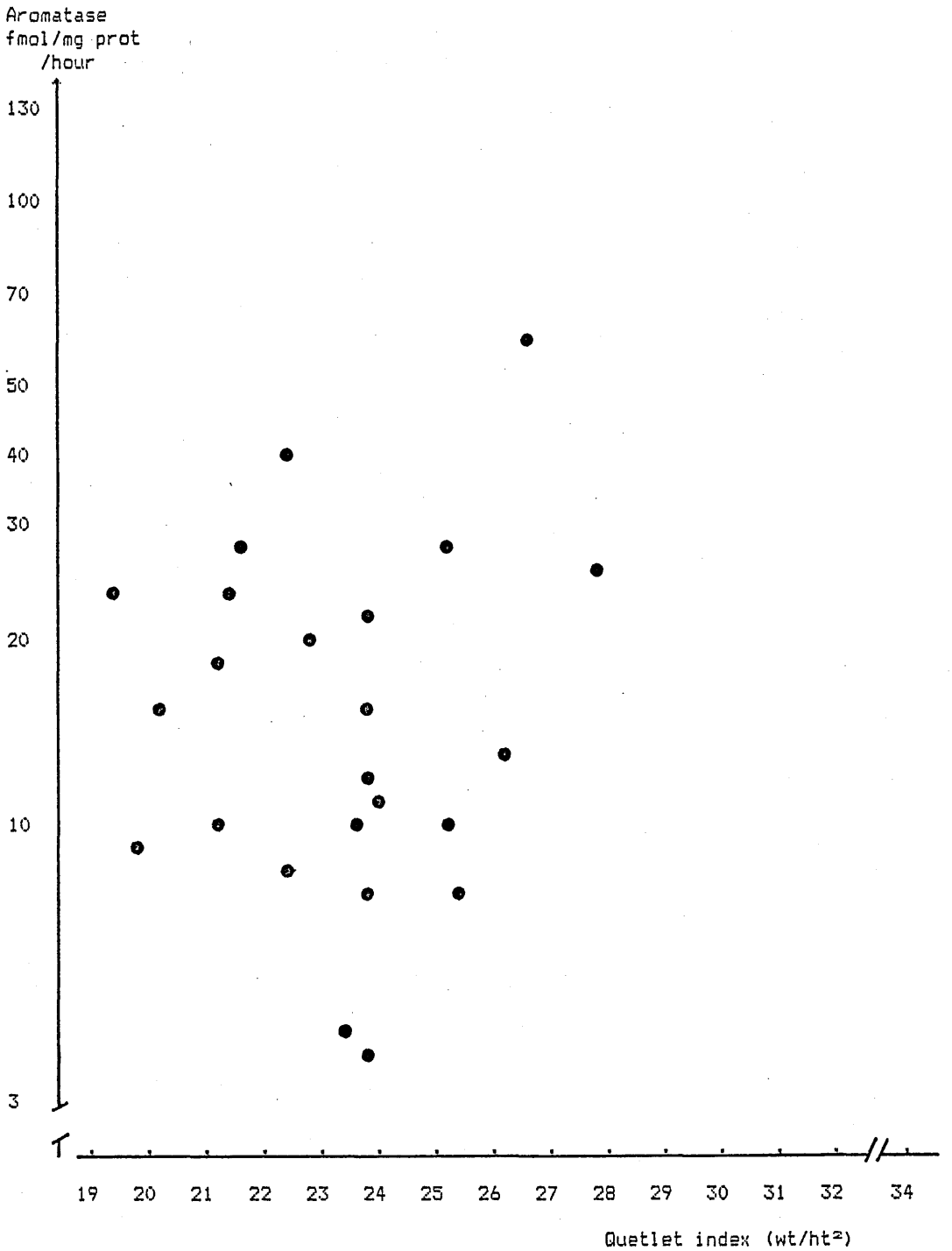



Figure 3:10b.

Adipose tissue aromatase activity and obesity (benign disease).

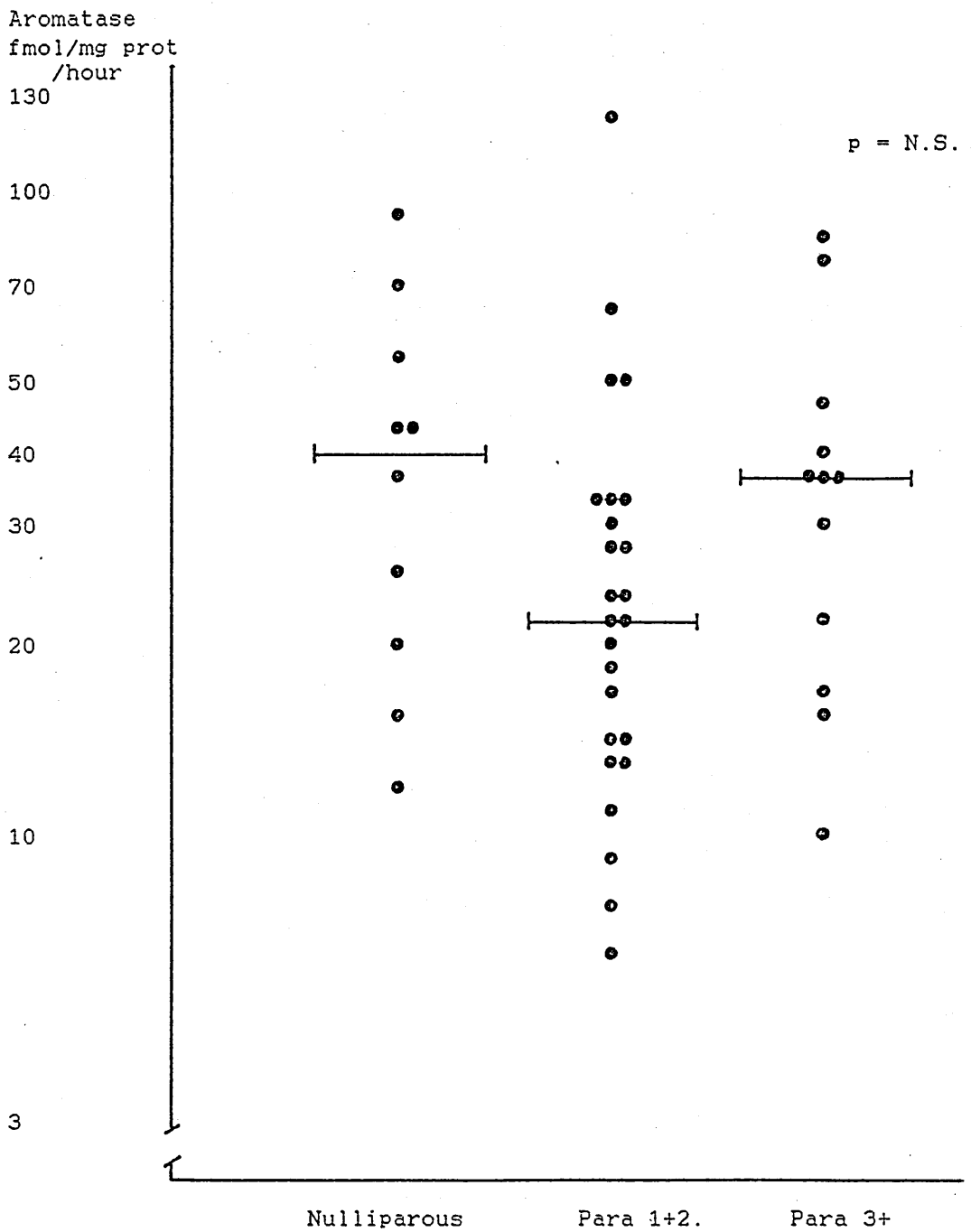



Figure 3:11a.

Adipose tissue aromatase activity and parity (cancer patients).

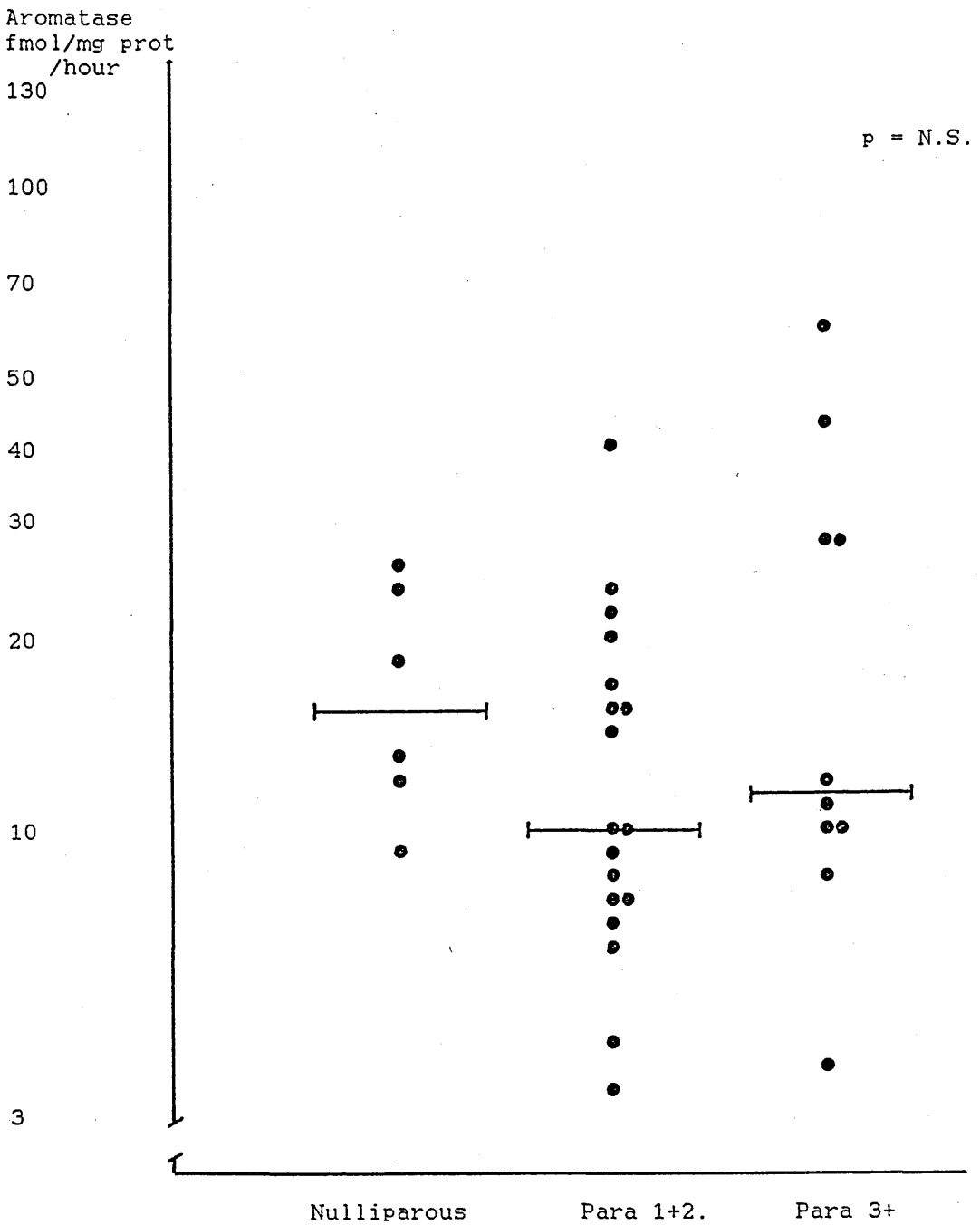



Figure 3:11b.

Adipose tissue aromatase activity and parity (benign disease).

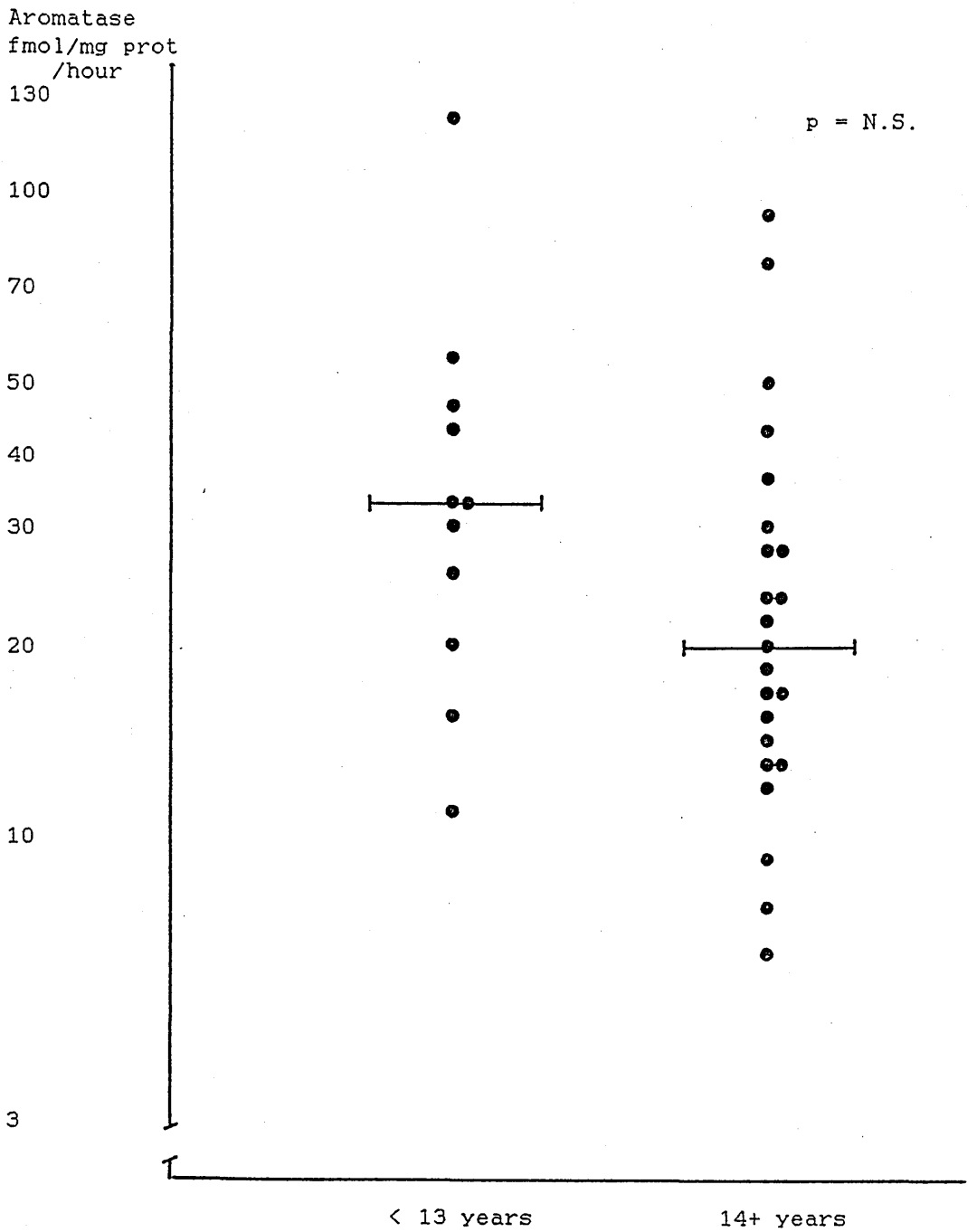



Figure 3:12a.

Adipose tissue aromatase activity and age at menarche (breast cancer).

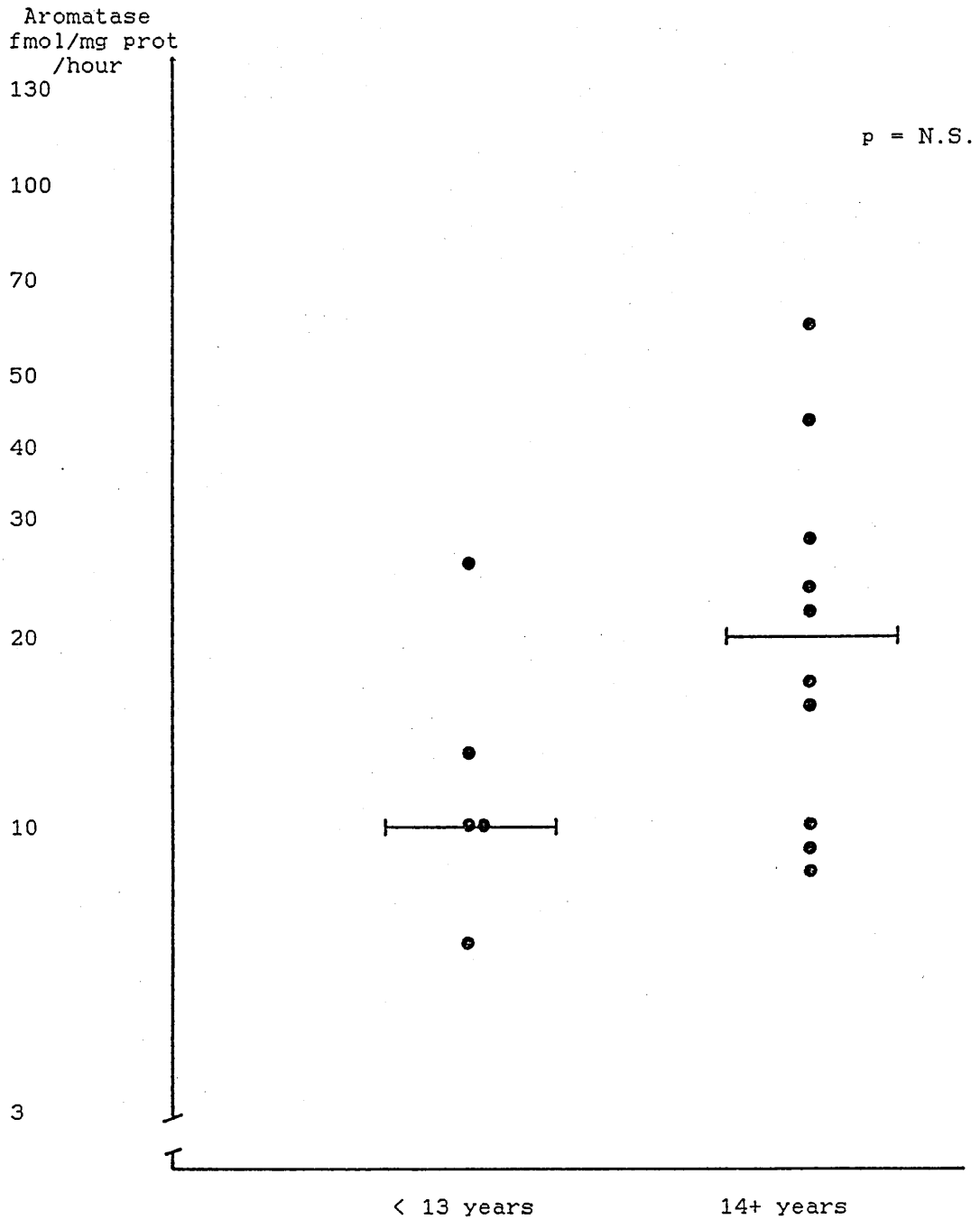



Figure 3:12b.

Adipose tissue aromatase activity and age at menarche (benign disease).

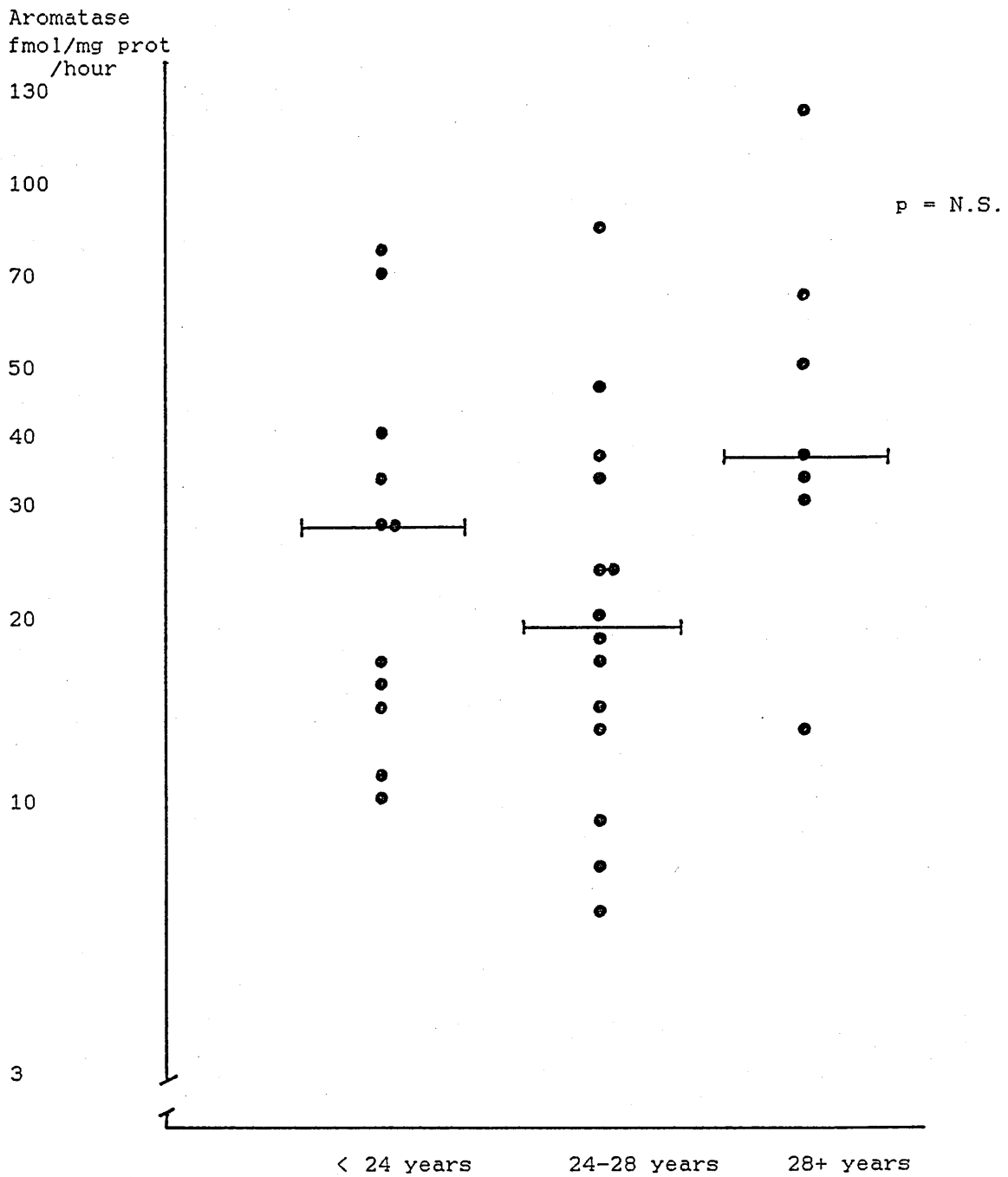



Figure 3:13a.

Adipose tissue aromatase activity and age at first pregnancy (breast cancer).

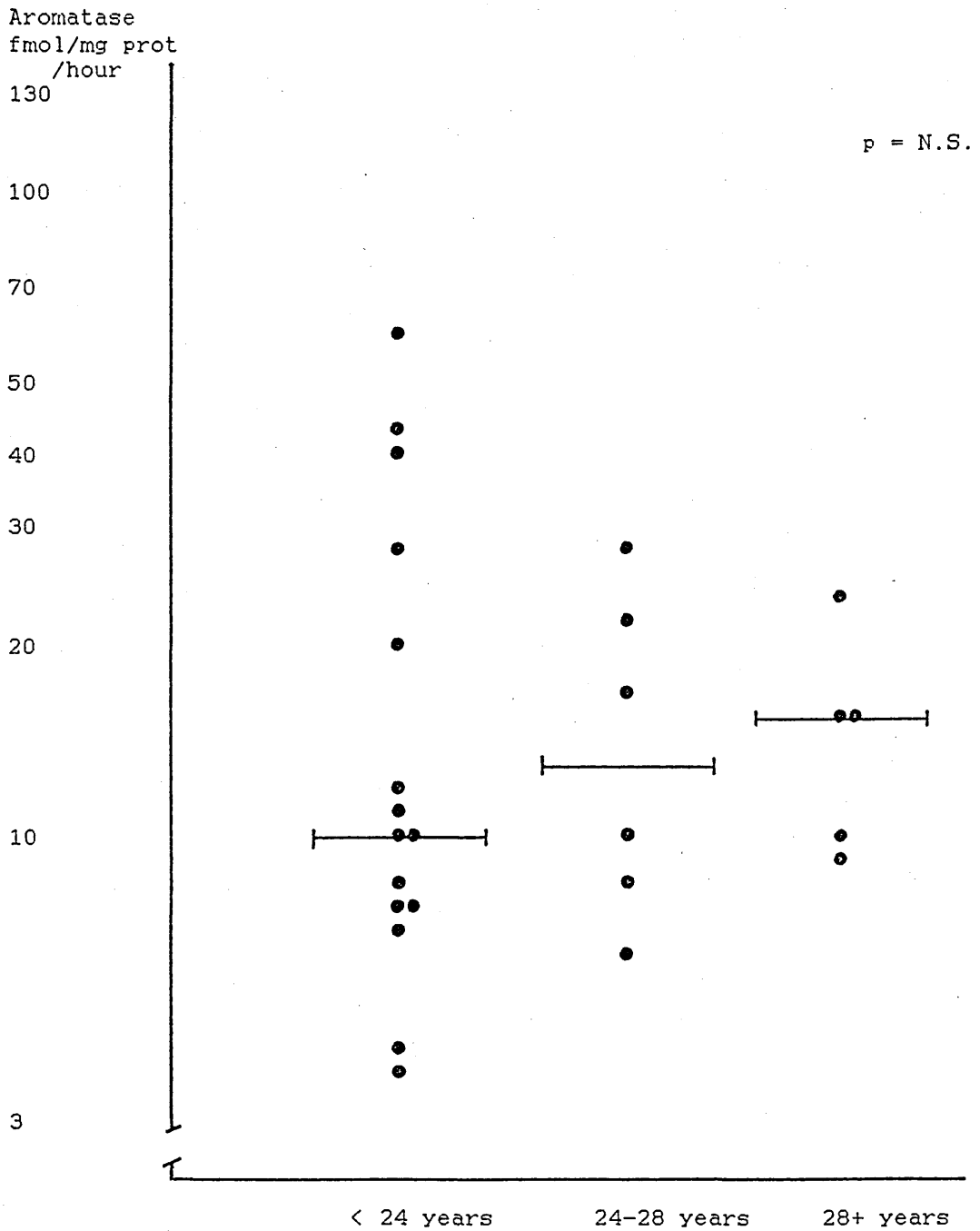



Figure 3:13b.

Adipose tissue aromatase activity and age at first pregnancy (benign disease).

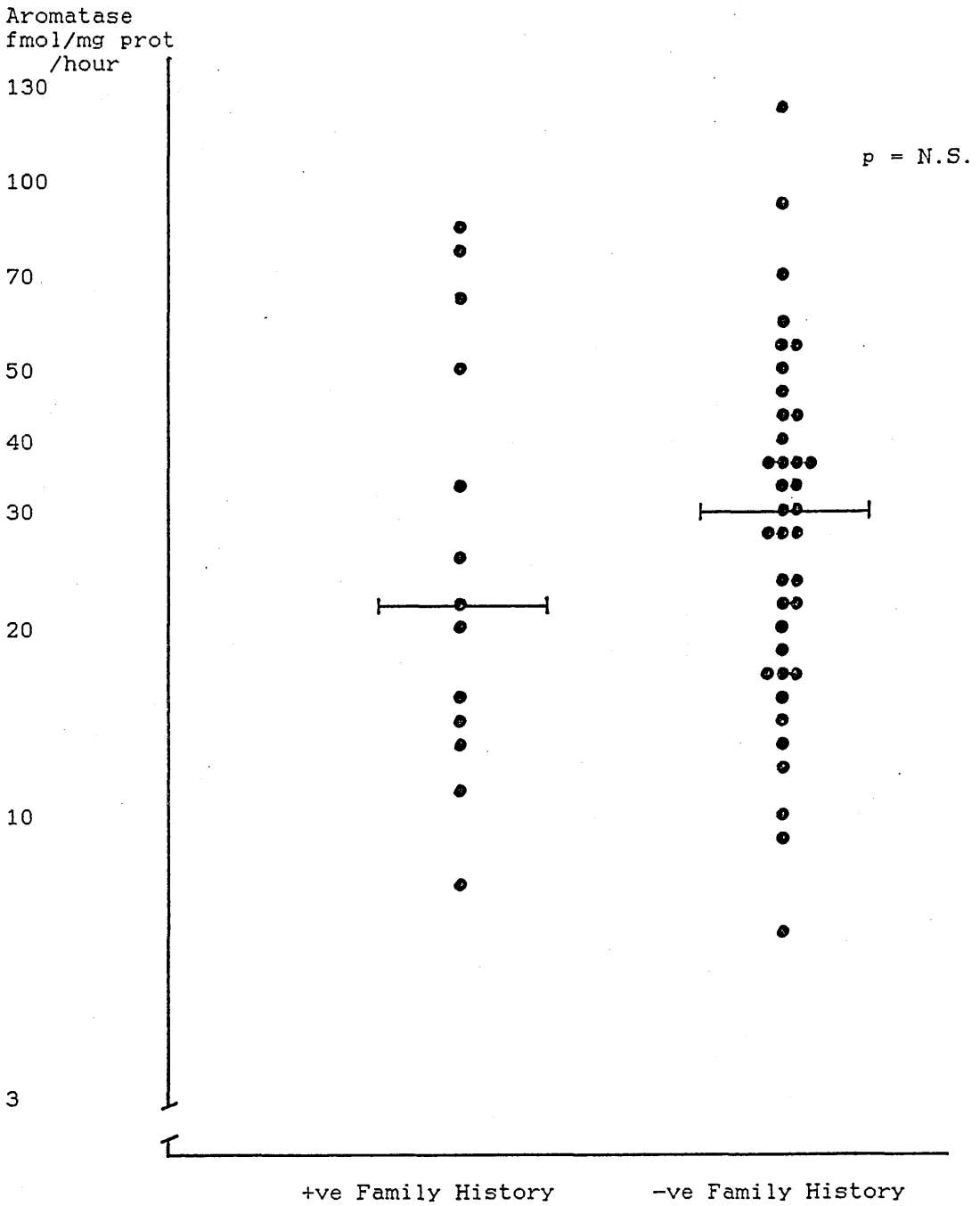



Figure 3:14a.

Adipose tissue aromatase activity and family history of breast cancer (cancer patients).

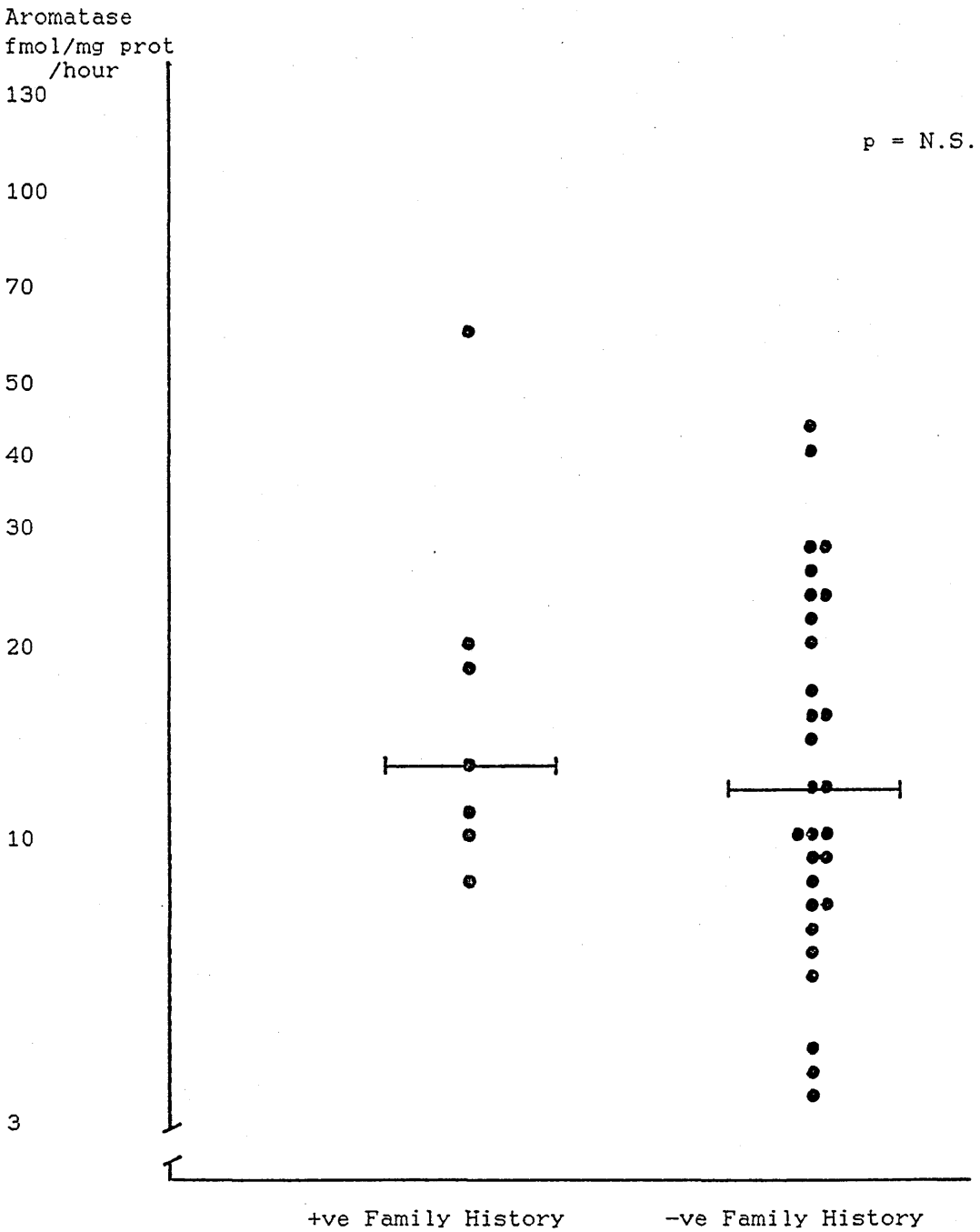



Figure 3:14b.

Adipose tissue aromatase activity and family history of breast cancer (benign disease).

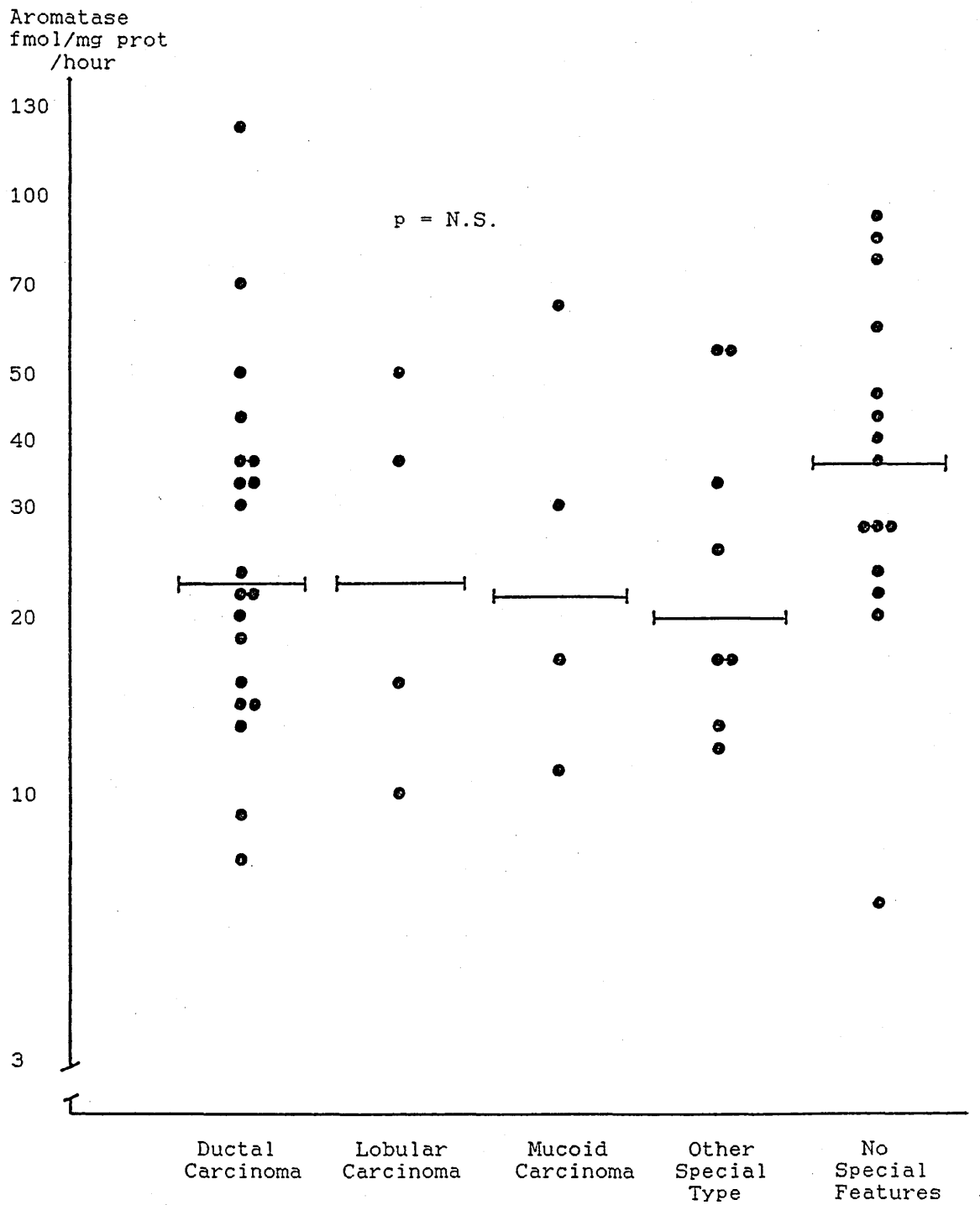



Figure 3:15.

Adipose tissue aromatase activity and tumour type.

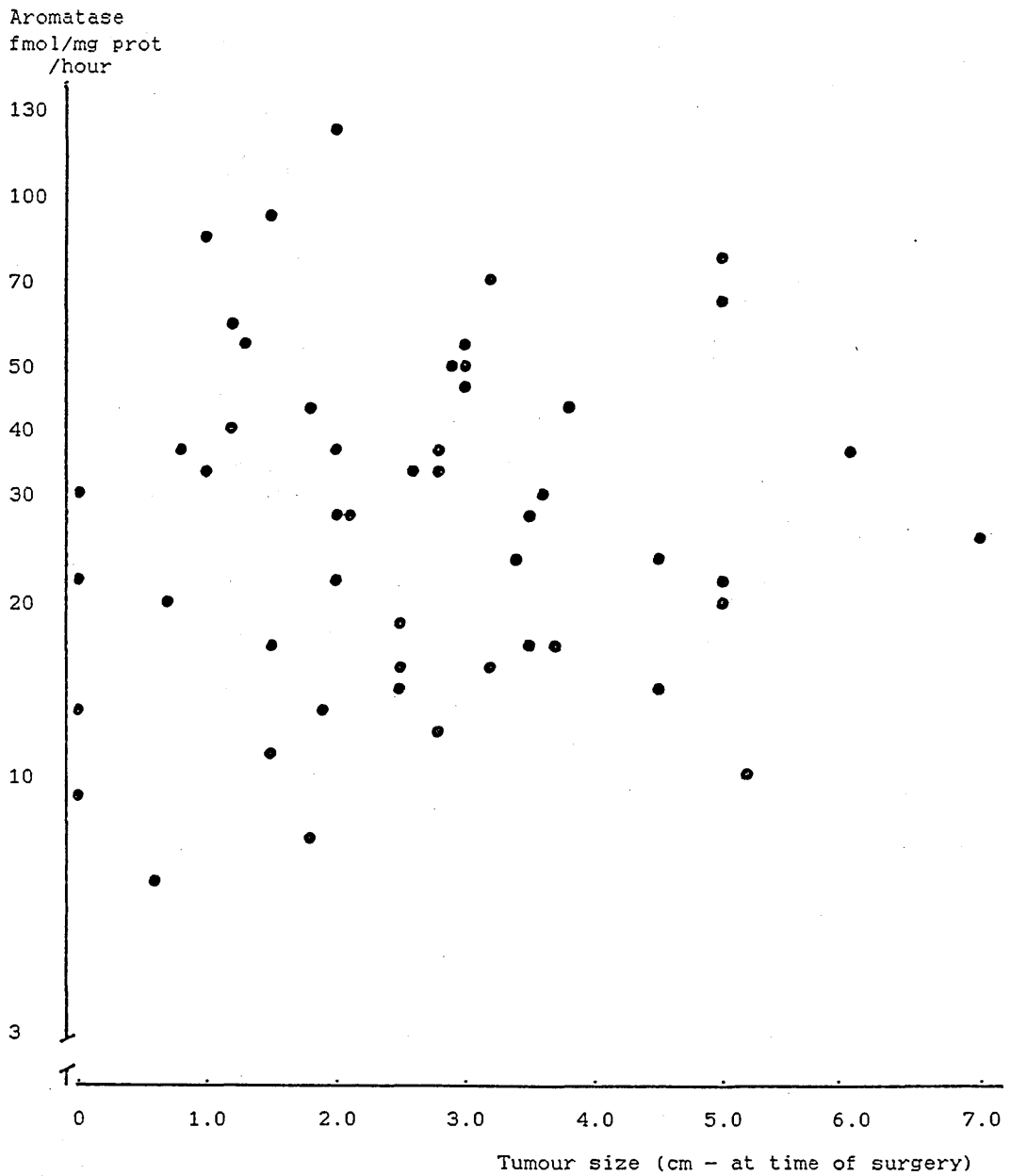



Figure 3:16.

Adipose tissue aromatase activity and tumour size.

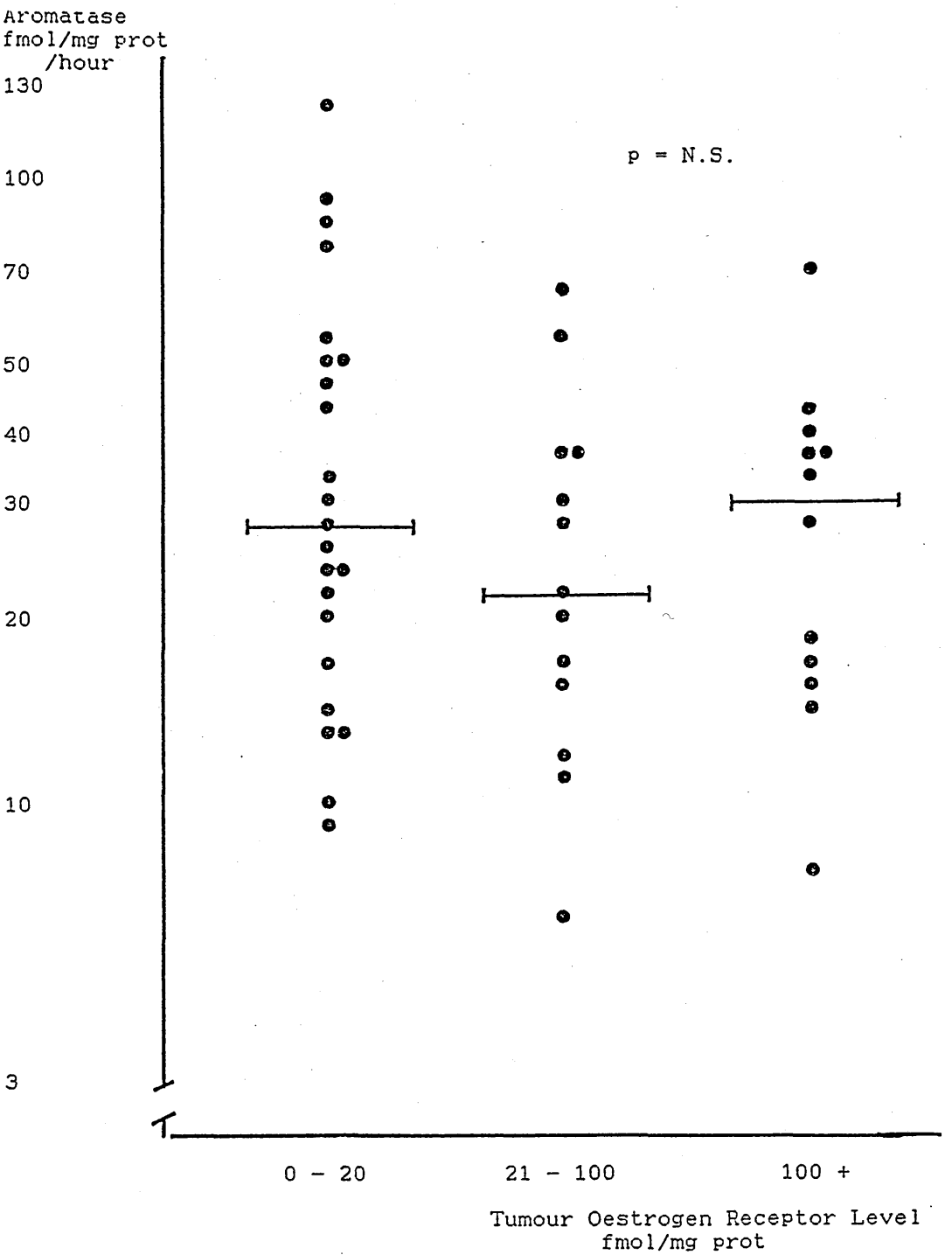



Figure 3:17.

Adipose tissue aromatase activity and tumour oestrogen receptor levels.

Discussion.

This study demonstrates the potential for oestrogen biosynthesis in all specimens of breast adipose tissue examined. Levels were of a similar magnitude to those reported by others in adipose tissue from various body sites[37,55,57,115,121].

An important observation was that despite the large variation in levels of aromatase activity between different specimens of adipose tissue, activity was significantly higher in tissue obtained from women with breast cancer compared with that in adipose tissue from women with benign lesions. Others have failed to detect such a difference[115,121] but have studied very small numbers of patients. The larger study of Beranek and co-workers also reported no significant difference between cancer and control patients but employed less sensitive assay conditions utilising soluble rather than particulate fractions[10]. Nevertheless aromatase activity was detected in 82% of cancer cases compared with only 50% of those with benign conditions suggesting a trend towards enhanced aromatase activity in adipose tissue from breast cancer patients.

There are two possible explanations for the presence of higher aromatase in breast adipose tissue from breast cancer patients, namely, either (1) malignant tumours are capable of stimulating aromatase activity in surrounding tissues or (2), regionally enhanced aromatase activity in adipose tissue produces a local environment which promotes malignant growth at that site.

With regard to the former possibility recent data suggest that growth factors enhance aromatase activity in peripheral tissues[100].

These include factors which may be produced in a paracrine manner by tumours[93] and preliminary results indicate that the addition of extracts from breast cancers added to cultures of adipose tissue may stimulate oestrogen biosynthesis[130].

Since oestrogens are heavily implicated in the promotion of breast cancer[91,154] local areas of enhanced oestrogen production might be expected to encourage development of tumour foci at these favourable sites. If this is the case one would expect a particular association between high aromatase activity in adipose tissue and oestrogen receptor positive tumours which are more likely to be oestrogen responsive. However such an association was not found in the present study. The possibility cannot be excluded, however that the tumours which were oestrogen receptor negative at the time of surgery were oestrogen receptor positive at an earlier stage in their evolution and have now become oestrogen independant.

As breast cancers in general have higher aromatase activity than adipose tissue[1,123] the higher activity in adipose tissue from breast cancer patients could also be due to the presence of micrometastatic deposits of malignant cells within the adipose tissue. Whilst this possibility cannot be completely excluded, portions of fat adjacent to that used for the aromatase assay were not shown to be involved microscopically with cancer.

Although levels of aromatase activity were higher in fat from breast cancer patients, a large range of values was found in patients with and without malignant breast disease. It is clear

therefore that other factors must influence the aromatase system. Statistically significant relationships were not observed between aromatase activity and age or menopausal status of the patients from whom the samples were obtained, although the highest levels of activity were observed in women aged between 40 and 55. This trend is similar to that noted in a study of abdominal adipose tissue[55]. However in view of the relative excess of pre-menopausal women in our benign group as compared with cancer patients and the reports of others that peripheral aromatase increases with age[28,57], the data was reanalysed excluding post-menopausal women. The difference in aromatase activity between the cancer patients and the non-malignant group remained highly significant, so it is therefore extremely unlikely to be an age related phenomenon.

There was no significant relationship between aromatase activity and various factors which are associated with excess risk of breast cancer such as height, weight, obesity , parity, age at menarche, age at first full term pregnancy, or family history of breast cancer. The differences between cancer and control patients are therefore unlikely to be due to any excess of these risk factors in the cancer group. Factors which influence the inherent level of aromatase in breast adipose tissue still therefore require to be identified.

# Chapter 4.

## Comparison of aromatase activity in breast adipose tissue with adipose tissue from other body sites.

## Introduction

If steroid metabolism in breast adipose tissue is of relevance in the evolution of breast cancer then levels of aromatase activity in the breast should at least be comparable with the activity found in other body sites. It has been suggested that activity in breast adipose tissue is lower than activity elsewhere in the body[10]. In that study, however, comparisons were made between samples from different individuals. The aim of this investigation was to compare aromatase activity in breast adipose tissue with that in samples from other sites in the same individual.

## Methods.

Adipose tissue was obtained simultaneously from the breast and from another body site in six patients undergoing other surgical procedures in addition to breast surgery. The sites from which tissues were obtained are shown in Table 4:1.

## Results:

The results are shown in Table 4:1. Levels of aromatase activity are comparable when expressed in terms of activity per gram of adipose tissue. Extracts of adipose tissue from other sites contained higher concentrations of protein suggesting that these samples were more cellular. The breast samples were more active if results are expressed in terms of activity per milligram of protein in the tissue extracts. (Figure 4:1, $p < 0.05$)

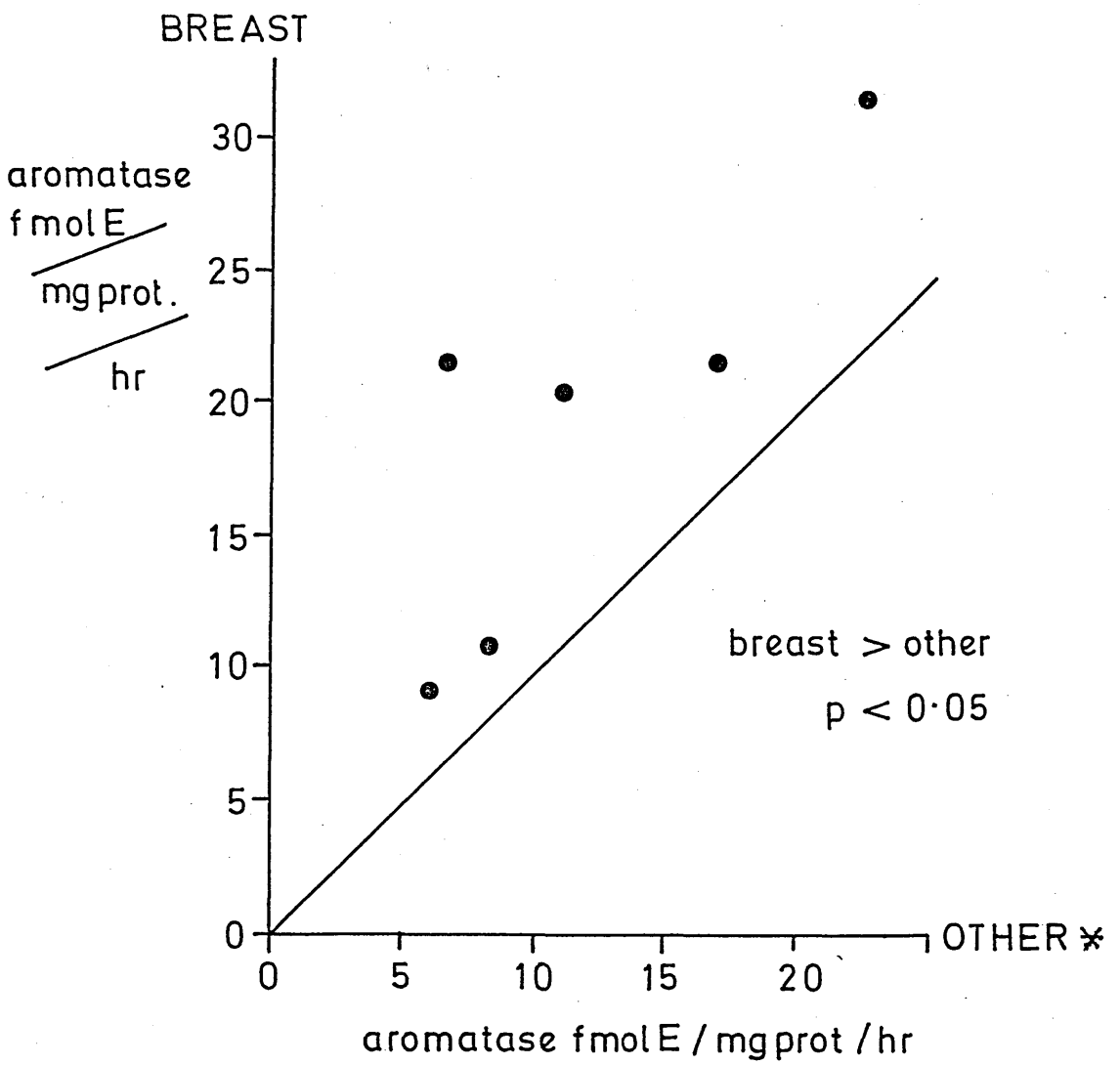
## Conclusion:

Although this is a small series of patients the results indicate that breast adipose tissue is as active as tissue elsewhere in the body in terms of aromatase activity.

Table 4:1.

Aromatase activity in breast adipose tissue compared with adipose tissue elsewhere in the body.

| No. | SITE | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | AROMATASE %CONV. | AROMATASE fmol/mg prot /hour |
|---|---|---|---|---|---|
| 11 | BREAST | 2 | 3.1 | .145 | 32.2 |
| | BACK | 1.2 | 3.2 | .109 | 22.7 |
| 12 | BREAST | 2 | 2.0 | .033 | 11 |
| | OMENTUM | 2 | 6.2 | .077 | 8.3 |
| 30 | BREAST | 2 | 2.6 | .036 | 9.2 |
| | AXILLA | 2 | 5.7 | .052 | 6.0 |
| 32 | BREAST | 2 | 1.6 | .053 | 22.1 |
| | BACK | 2 | 2.5 | .064 | 17.1 |
| 46 | BREAST | 1.6 | 1.4 | .046 | 21.9 |
| | BACK | 1.6 | 5.0 | .051 | 6.8 |
| 49 | BREAST | 2 | 2.2 | .067 | 20.3 |
| | BACK | 2 | 5.0 | .085 | 11.3 |




* back - 4 , omentum - 1, axilla - 1

Figure 4:1

Aromatase activity in breast adipose tissue compared with other body sites.

## Chapter 5.

# Aromatase activity in adipose tissue from breast quadrants of patients with breast cancer.

## Introduction

The results in Chapter 3 suggest that breast cancer is associated with enhanced aromatase activity in adjacent adipose tissue. Breast cancers occur predominantly in the upper outer quadrant of the breast[70]. It remains to be determined whether aromatase activity in adipose tissue varies throughout individual breast quadrants. The aims of this study were therefore to: a) investigate if aromatase activity varies between different quadrants of the same breast by measuring activity in adipose tissue from the mastectomy specimens of patients with breast cancer, and b) determine if there is any consistent pattern of distribution of aromatase activity in relation to the quadrants of the breast or the location of the tumours.

## Materials & methods

### Tissues:

Adipose tissue was obtained from the four quadrants of twelve consecutive mastectomies performed for histologically confirmed breast cancer and carefully separated from breast parenchyma or fibrous tissue. (Patient numbers are given in Table 5:1, further details of these patients are included in Appendix Tables A1 and A3.)

### Aromatase assay

Particulate fractions from the adipose tissue samples were incubated in tritium release assays as previously described.

Statistical methods

Aromatase levels in the four breast quadrants were compared using the Friedman test, and average levels in the tumour-bearing quadrants were compared to average levels in quadrants not bearing tumours by the Wilcoxon signed-ranks test. Multiple regression analysis on the logarithms of the 48 aromatase activities was used to test the combined association of tumour and anatomical location on the aromatase level. The analysis treated patient, location and presence or absence of tumour as fixed effects with 12, 4, and 2 levels respectively, and these tests were performed for the significance of location, controlling for patient and tumour, and of tumour controlling for patient and location.

Results:

Aromatase activity was detected in all samples studied (range 3.6 - 35 units) Considerable variation was observed between individuals (average values 6.3 - 26.9 units) and between different quadrants in the same breast, the ratio of activity in the most to the least active quadrants ranging from 1.4 to 4.9 (Table 5:1 - full details of the results are given in Appendix Table B5). There was a significant association between the anatomical location of the quadrant and levels of aromatase activity ($p < 0.05$). The highest activity was present in the upper outer quadrant in seven cases, the lower outer quadrant in three and the upper inner quadrant in two. The lower inner quadrant was never the site of highest activity but represented the least activity in five cases. The upper inner quadrant held the least activity in four cases and the lower outer quadrant the remaining three. The upper outer quadrant never exhibited

TABLE 5:1. Adipose Tissue Aromatase Activity in Breast Quadrants
(Figures in brackets - ratio of activity to lowest activity in that breast)

| Patient | Aromatase Activity in Breast Quadrants | | | | |
|---|---|---|---|---|---|
| | LIQ | LOQ | UIQ | UOQ | Mean |
| 4. | 10.5* (1.7) | 15.0* (2.4) | 6.2 (1) | 9.5 (1.5) | 10.3 |
| 9. | 7.7 (1.1) | 7.1 (1) | 34.9* (4.9) | 12.3 (1.7) | 15.5 |
| 11. | 22.9 (1) | 27.4 (1.2) | 24.9 (1.1) | 32.2* (1.4) | 26.9 |
| 14. | 26.0 (1.1) | 23.9 (1) | 23.6 (1) | 32.0* (1.4) | 26.4 |
| 15. | 15.0 (1.4) | 35.0* (3.3) | 10.5 (1) | 17.8* (1.7) | 19.6 |
| 18. | 3.6 (1) | 3.6 (1) | 10.8* (3.0) | 7.2* (2.0) | 6.3 |
| 23. | 4.9 (1) | 10.3 (2.1) | 10.5 (2.1) | 23.7* (4.8) | 12.4 |
| 26. | 5.6 (1) | 7.1 (1.3) | 12.2 (2.2) | 14.0* (2.5) | 9.7 |
| 33. | 9.9 (1) | 19.0*+ (1.9) | 13.9*+ (1.4) | 13.1*+ (1.3) | 14.0 |
| 34. | 10.9 (2.1) | 5.2 (1) | 8.2 (1.6) | 15.9* (3.1) | 10.1 |
| 39. | 9.7 (1) | 12.3 (1.3) | 12.5 (1.3) | 15.4* (1.6) | 12.5 |
| 47. | 11.7 (1.9) | 14.0+ (2.3) | 6.2 (1) | 17.3* (2.8) | 12.3 |

* Quadrant containing clinically detected tumour
\+ Additional tumour foci discovered on histology

LIQ - Lower Inner Quadrant LOQ - Lower Outer Quadrant
UIQ - Upper Inner Quadrant UOQ - Upper Outer Quadrant

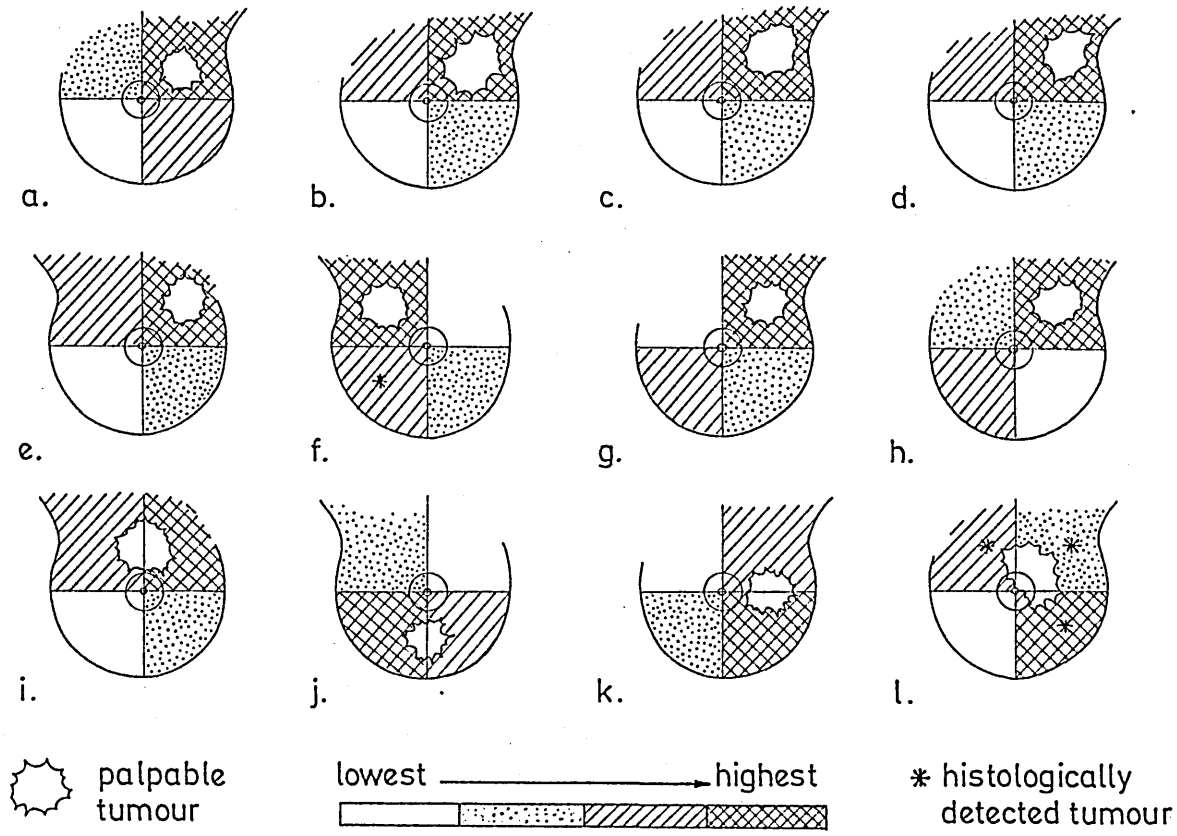



Figure 5:1. Adipose tissue aromatase activity in breast quadrants.

(Quadrants are shaded according to the level of aromatase activity as indicated in the key)

the lowest activity.

The relationship of activity to the sites of clinically and pathologically detected tumour is shown diagrammatically in Figure 5:1. In 8 cases the main tumour mass was located entirely in one quadrant: in all 8 this quadrant had the highest activity. In patient 47. a second focus of tumour was detected in the lower outer quadrant which had the second highest activity. In three cases the tumour mass straddled two quadrants both of which had higher activity than the non-involved segments. The remaining case had invasive tumour in three quadrants but no tumour in the quadrant with lowest activity.

Levels of activity were therefore strongly correlated with the presence of tumour in a quadrant: in all twelve cases the average level in the tumour-bearing quadrants was higher than in those not bearing tumours ($p < 0.001$). The mean ratio of aromatase activity in tumour bearing quadrants as compared to those not bearing tumours is estimated as 1.89, with 95% confidence limits of 1.56 and 2.31 . A multiple regression analysis reveals that whilst there was no significant difference in activity between the various quadrants after correction for the presence or absence of tumour, the activity was significantly correlated with the presence of tumour in a quadrant irrespective of its anatomical location ($t = 5.45$, 32 d of f, $p < 0.001$). No relationship was apparent between the average individual activity and the patient's height, weight or obesity, nor was there any link with tumour type, size or stage.

## Discussion

All samples of mammary adipose tissue again possessed aromatase activity but there was large variation in levels not only between different patients but also within individual breasts. Whilst the variation within the breast had some anatomical basis the level of activity was much more significantly related to the presence of tumour in a quadrant with activity in tumour-bearing quadrants being between one and a half to two and a half times the level in other quadrants. The trend for high activity to be present in the upper outer quadrant probably reflects the more frequent occurence of tumours in this location as seen in the present study and as reported elsewhere [70].

The increase in aromatase activity in tumour bearing quadrants is unlikely to be due to increased cellularity of the tissues in these quadrants. Increased cellularity would be reflected in a higher protein concentration of tissue extracts. The protein concentrations of samples were relatively consistent within individual breasts and were not highest in tumour-bearing quadrants.

Several reasons could account for the enhanced aromatase activity in quadrants containing tumours. Breast cancers in general show higher aromatase activity than adipose tissue [1,123], and it is possible that adipose tissue samples from tumour-bearing quadrants contain micrometastatic deposits of tumour. This cannot be totally excluded but there was no evidence of tumour deposits in samples of adipose tissue examined histologically. Furthermore the adipose

tissue was taken from the periphery of breast quadrants in areas remote from the palpable tumour in order to minimise the risk of including tumour foci within the samples.

Assuming that elevated aromatase activity in tumour bearing quadrants is not due to contamination of samples by tumour cells, the results would still be in keeping with the suggestion that regionally enhanced aromatase activity in adipose tissue may be producing a local environment which promotes malignant growth. Alternatively malignant tumours may be capable of stimulating metabolic activity in their surrounding tissues. This effect could be specific for the aromatase system or may be part of an increase in several enzymes. Such an effect could result from the production of specific substances which stimulate these particular enzymes or could be due to an indirect effect of tumours on their surrounding tissues, e.g. by increasing cellularity or vascularity. It should be possible to determine if there is a non-specific increase in metabolism around breast cancers by measuring activity of other enzymes in the same samples. It is possible to measure activity of other steroid metabolic pathways in these same incubations since the steroid metabolites are extracted into chloroform at the end of each incubation and these had been stored for further analysis.

# Chapter 6.

## 17-beta hydroxysteroid dehydrogenase activity in breast adipose tissue.

## Introduction

The results in chapters 3 & 5 suggest an association between elevated aromatase activity and breast cancer. A possible explanation for this phenomenon is that tumours stimulate an increase in metabolism in their surrounding tissues. It should be possible to determine if there is a general increase in metabolism by measuring activity of other enzymes in the same tissue samples.

Preliminary studies on steroid metabolism in adipose tissue (results not shown) indicated that metabolism of the substrate androstenedione to products other than oestrogen was occuring in these incubations. The most substantial metabolite (in terms of percentage conversion) was testosterone. Such metabolism has been noted in other studies[121,123]. This transformation is mediated by the 17-beta hydroxysteroid oxidoreductase enzyme(s) usually referred to as 17-beta hydroxysteroid dehydrogenase (17BHSD). This enzyme has a pivotal role in the interconversion of both androgens and oestrogens; transforming the relatively inactive precursors androstenedione and oestrone into the more biologically active testosterone and oestradiol. Previous studies on this enzyme in the breast have shown that levels of activity vary greatly between breast tumours, normal breast tissue and adipose tissue[10,14,16]. Since this enzyme could play a key role in modifying the local hormonal environment of the breast, it is of interest to study its activity in the breast adipose tissue samples.

The activity of this enzyme may be measured simultaneously in the tritium release assay since the steroid metabolites (including

testosterone) were extracted from the incubations into chloroform. The majority of these extracts were available for further analysis.

Methods

Chloroform extracts from the incubations of adipose tissue (performed as described in Chapters 3 & 5) were retained and stored at -20 $^{o}$C until assayed (45 cancer patients, 32 benign disease). To monitor procedural losses, radioinert testosterone (250 ug) was added to each sample. Aliquots were taken from each extract for determination of the total radioactivity present (by scintillation counting) The samples were then evaporated to dryness under a continuous stream of nitrogen, reconstituted in 200 ul of ethanol and run in 2 consecutive Thin Layer Chromatography systems (ref): Chloroform: Acetone 185:15 followed by Benzene: Ethyl Acetate 80:20. This separates testosterone from the other steroid metabolites; in particular the radiolabeled androstenedione. The testosterone fractions were then characterised by chemical derivative formation using the following techniques:-

Acetylation

Acetic anhydride (6 drops) and pyridine (3 drops) were added to the dried steroid, which was then incubated at 60 $^{o}$C for one hour. Methanol (1ml) was added and the mixture re-evaporated to dryness. The reaction products were then purified by further thin layer chromatography (cyclohexane:ethyl acetate 70:30) and the testosterone acetate fraction recovered.

Acetate hydrolysis

Testosterone acetate fractions were dried, dissolved in methanol

(1ml) containing 2% Potassium Carbonate (0.25ml) and incubated overnight at 37 $^{o}$C. Distilled water (3ml) was added and the steroid extracted into ethyl acetate (5ml x 2) centrifuging to partition. The ethyl acetate extracts were then bulked, dried and purified by thin layer chromatography (Chloroform: acetone 185:15) and the free testosterone recovered.

After the acetylation and hydrolysis reactions the purified testosterone acetate and testosterone fractions were dissolved in a measured volume of ethanol. The radioactivity in a standard aliquot was measured by liquid scintillation counting. The quantity of recovered steroid was calculated by measuring the optical density of the solution at 281nm (Unicam SP800) against known standards. (Correction for non-specific absorbance was acheived by subtracting the readings from other areas on the tlc plate.) The specific activity was calculated as shown below:

$$\text{specific activity} = \frac{\text{dpm in sample}}{\text{amount of steroid recovered}} \quad \text{(dpm/nmol)}$$

Purification by thin layer chromatography and chemical derivative formation continued until three successive specific activities were consistent to within 10%.

The percentage conversion of androstenedione to testosterone was then calculated as shown below:

$$\text{Percentage conversion} = \frac{\text{specific activity x cold steroid added (nmol) x 100}}{\text{total dpm in original sample}}$$

Since the specific radioactivity of the androstenedione was known, the percentage conversion could be converted to pmol and then expressed as pmol testosterone / mg protein / hour (units).

Results:

Radiolabeled testosterone was detected in all of the extracts examined indicating that 17BHSD activity was present in all cases.

The individual results are listed in Tables B:3-B:5 (Appendix B). Again wide variation in levels of activity has been found between individuals (0.03 - 52.8 units) and between the quadrants of individual breasts (up to six-fold difference between the most and least active quadrants). Figure 6:1 compares the activity in breast cancer patients with benign disease patients. There is no significant difference in levels of activity between cancer patients [median 1.4 (0.03 - 52.8) units] and non-cancer patients [median 1.0 (0.1 - 10.3) units].

The levels of activity found in the quadrants of the twelve mastectomies are shown in Table 6:1 and illustrated diagramatically in Figure 6:2. There is no consistent pattern of distribution of activity within the breast quadrants (Friedman Test) or in relation to tumour site (Wilcoxon signed rank test).

Figure 6:3 shows enzyme activity related to tumour size at the time of surgery for all patients and Figure 6:4 for post-menopausal women only. In the post-menopausal group there is a significant correlation between tumour size and enzyme activity ($r = 0.71$ $p < 0.01$). This relationship does not reach statistical significance in the larger group ($r = 0.25$). Nevertheless the trend for higher activity to be associated with larger tumours is confirmed ($p < 0.01$ by Wilcoxon rank test) if levels of activity around tumours greater than 3cm are compared with smaller lesions (Figure 6:5).

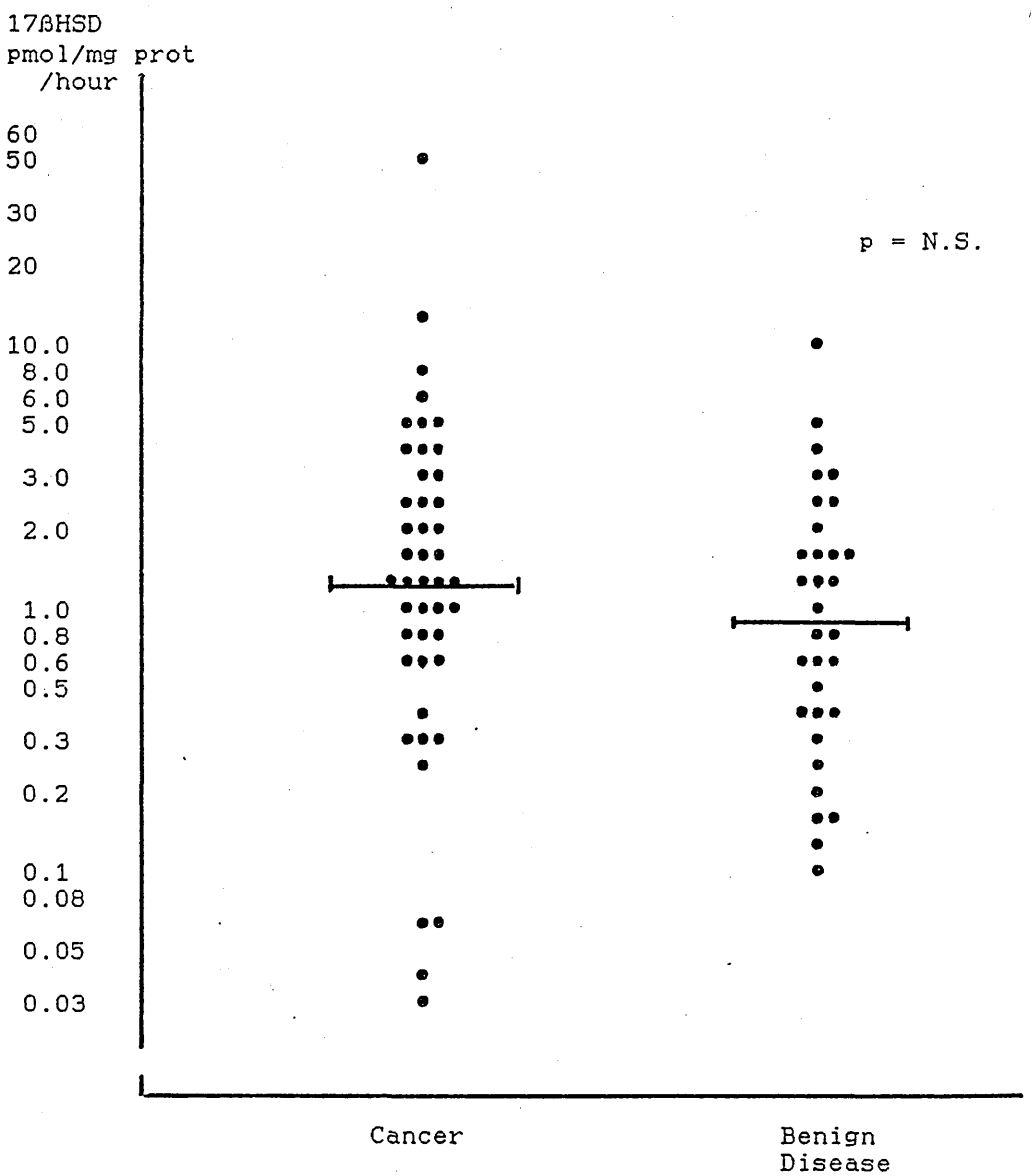



Figure 6:1.

Adipose tissue 17ßHSD activity in patients with breast cancer and benign breast disease.

TABLE 6:1. Adipose Tissue 17BHSD Activity in Breast Quadrants
(Figures in brackets - ratio of activity to lowest activity in that breast)

| Patient | 17BHSD Activity in Breast Quadrants | | | | |
|---|---|---|---|---|---|
| | LIQ | LOQ | UIQ | UOQ | Mean |
| 4. | 1.9* (1.3) | 1.6* (1.1) | 1.5 (1) | 1.7 (1.1) | 1.7 |
| 9. | 1.9 (2.7) | 1.2 (1.7) | 3.6* (5.1) | 0.7 (1) | 1.9 |
| 11. | 0.06 (1) | 0.07 (1.2) | 0.07 (1.2) | 0.07* (1.2) | 0.07 |
| 14. | 0.57 (1.2) | 0.9 (1.9) | 0.47 (1) | 0.5* (1.1) | 0.6 |
| 15. | 0.4 (1) | 1.7* (4.3) | 0.5 (1.3) | 0.5* (1.3) | 0.8 |
| 18. | 0.56 (1) | 0.7 (1.2) | 0.9* (1.6) | 0.87* (1.5) | 0.8 |
| 23. | 4.13 (1) | 4.18 (1) | 5.8 (1.4) | 4.1* (1) | 4.6 |
| 26. | 0.07 (2.3) | 0.08 (2.7) | 0.03 (1) | 0.06* (2.0) | 0.06 |
| 33. | 0.04 (1) | 0.05*+ (1.3) | 0.04*+ (1) | 0.07*+ (1.8) | 0.05 |
| 34. | 2.0 (1.1) | 3.7 (1.9) | 2.2 (1.2) | 1.9* (1) | 2.5 |
| 39. | 0.37 (1.2) | 0.39 (1.3) | 0.36 (1.2) | 0.3* (1) | 0.36 |
| 47. | 4.3 (3.6) | 1.2+ (1) | 2.3 (1.9) | 6.6* (5.5) | 3.6 |

* Quadrant containing clinically detected tumour
\+ Additional tumour foci discovered on histology

LIQ - Lower Inner Quadrant LOQ - Lower Outer Quadrant
UIQ - Upper Inner Quadrant UOQ - Upper Outer Quadrant

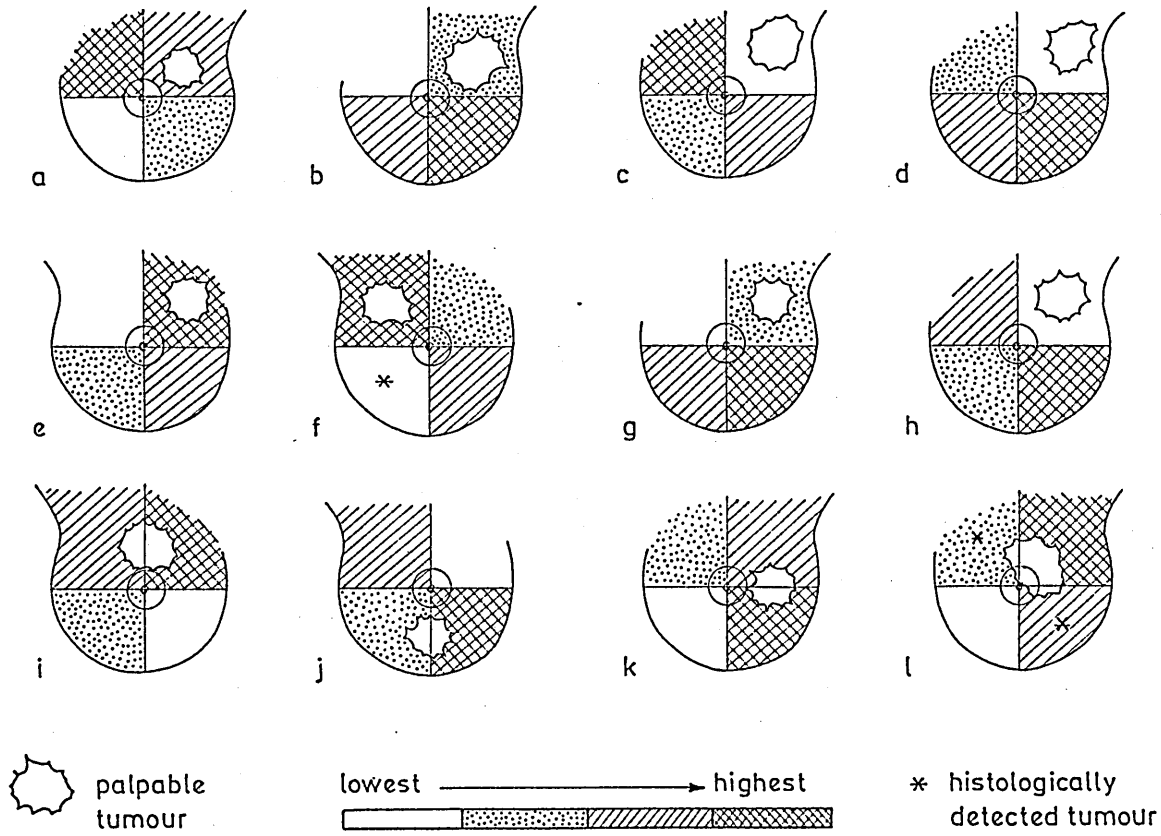



Figure 6:2. Adipose tissue 17BHSD activity in breast quadrants.

(Quadrants are shaded according to the level of 17BHSD activity as indicated in the key.)

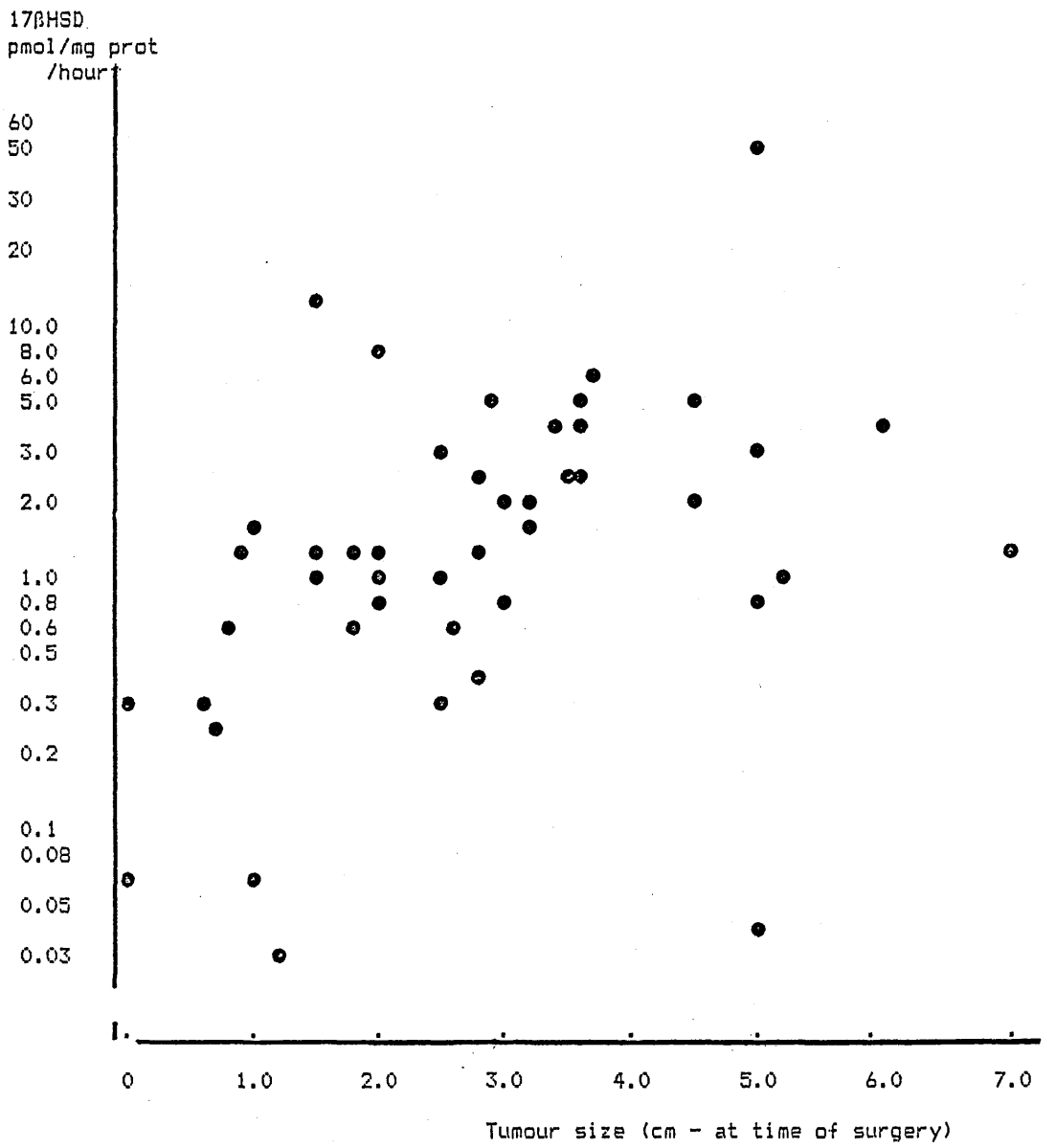



Figure 6:3.

Adipose tissue 17ßHSD activity and tumour size (all cases).

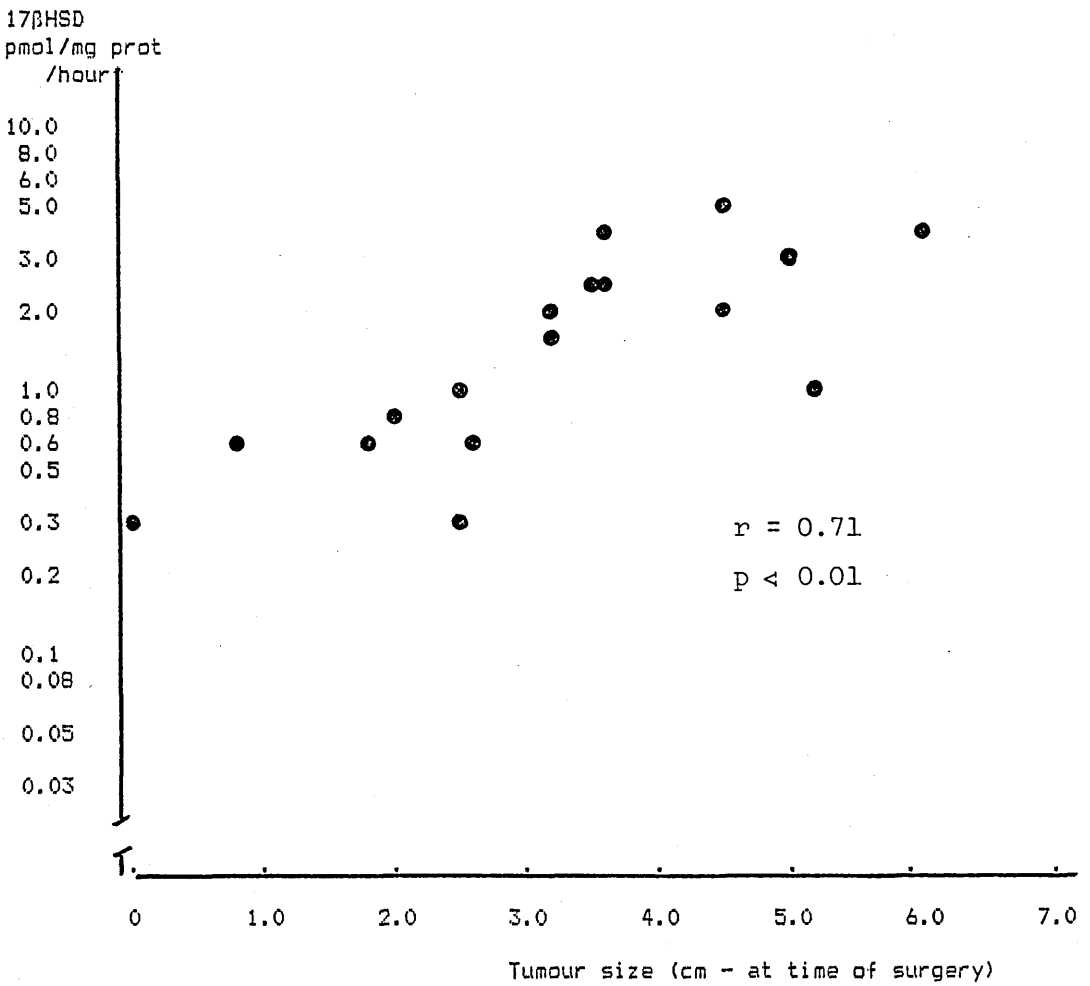



Figure 6:4.

Adipose tissue 17βHSD activity and tumour size (post menopausal only).

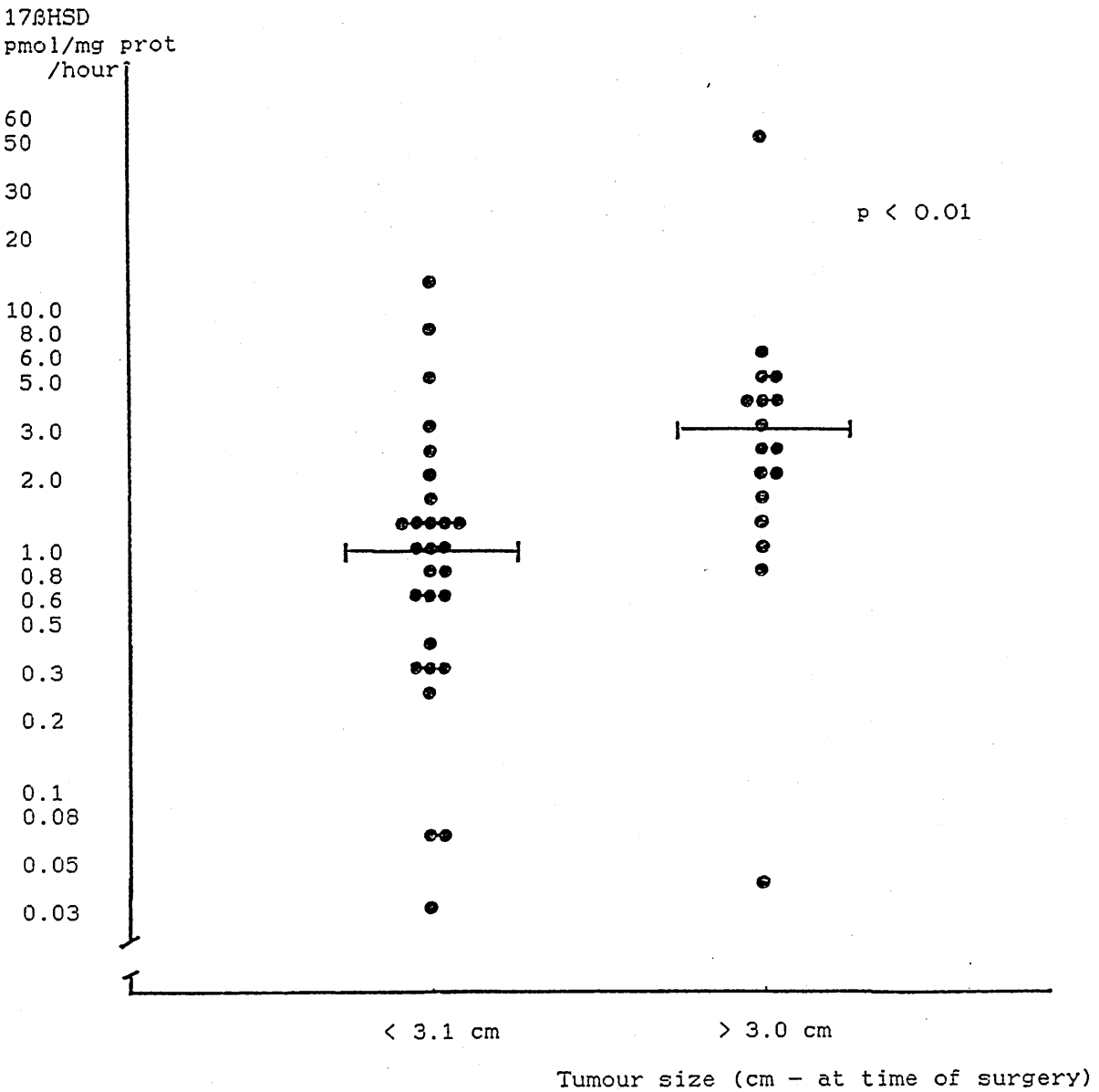



Figure 6:5.

Adipose tissue 17ßHSD activity and tumour size.

Figure 6:6 relates levels of activity to the presence or absence of nodal metastases for the 41 patients who had axillary staging performed (minimum of 4 nodes examined). Activity is significantly higher in those patients with nodal metastases (n = 17, median 2.1 units) compared to node negative patients (n = 24, median 0.8 units, $p < 0.01$).

Figures 6:7-6:10 show levels of activity plotted against age, obesity, parity (parous v nulliparous) and menopausal status showing that there is no apparent relationship between activity and any of these factors.

Analysis of results in relation to patient height, weight and family history of breast cancer also failed to reveal any significant correlation (results not shown).

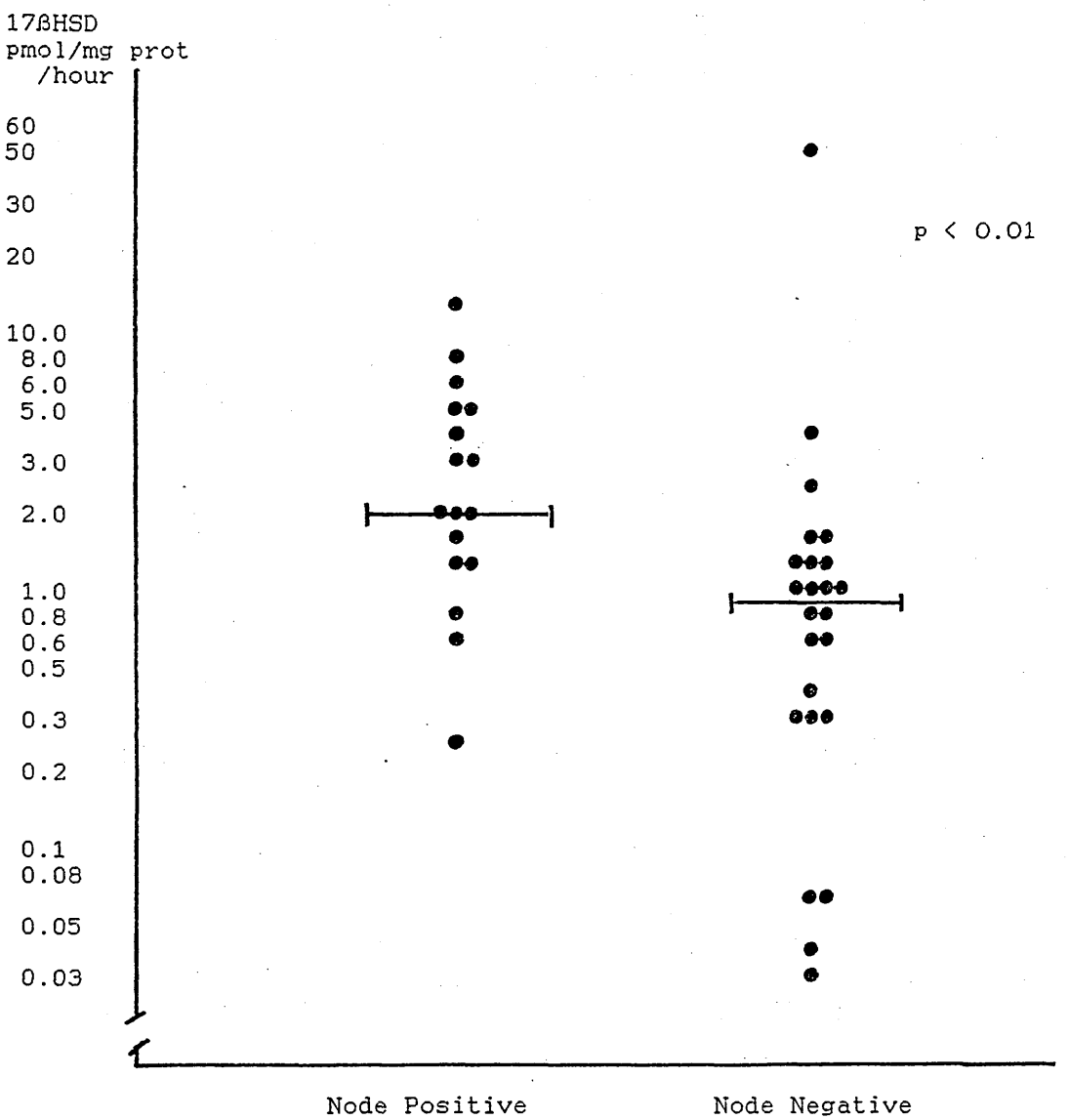



Figure 6:6.

Adipose tissue 17ßHSD activity and nodal metastases.

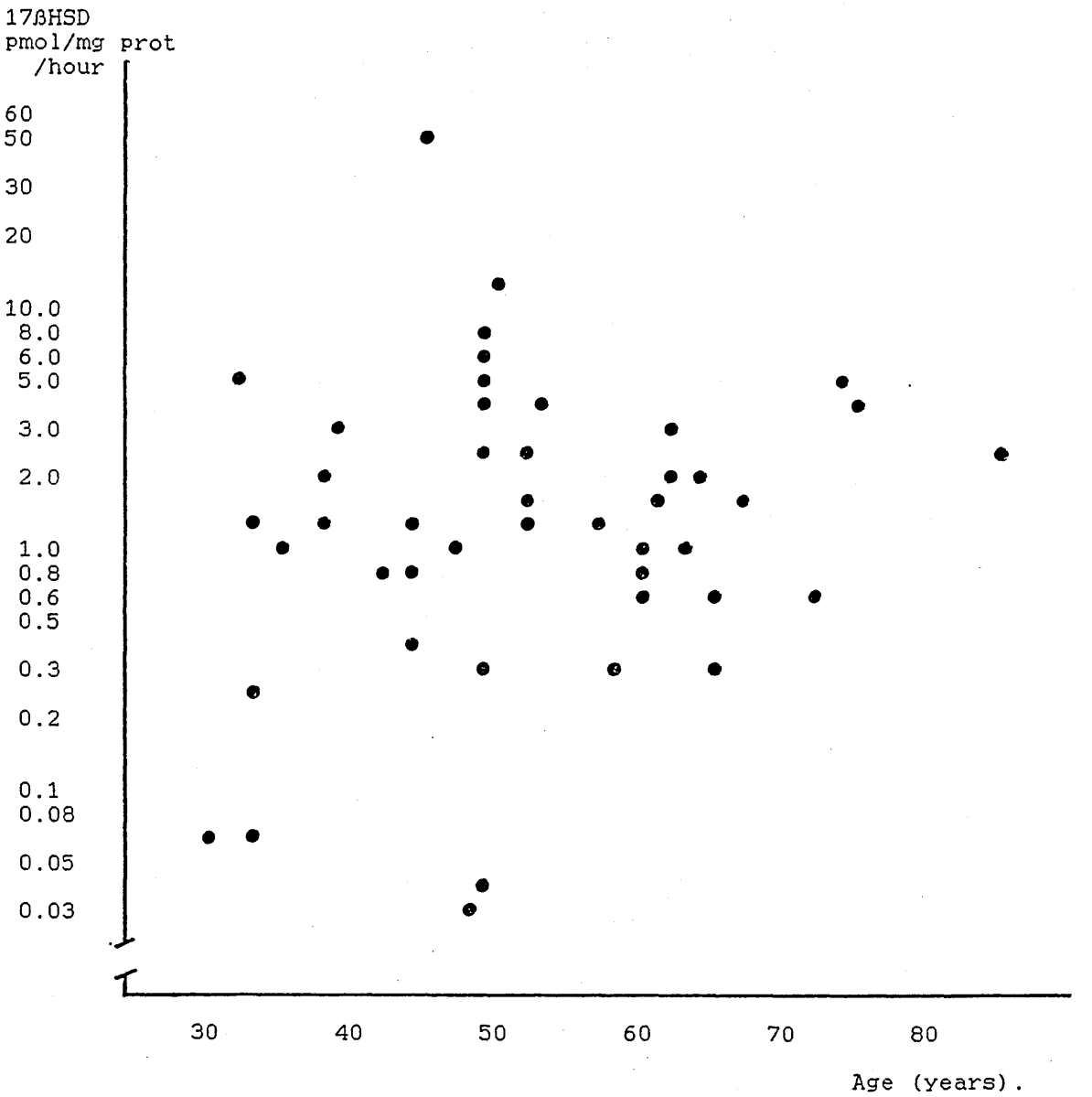



Figure 6:7a.

Adipose tissue 17ßHSD activity and patient age (cancer patients).

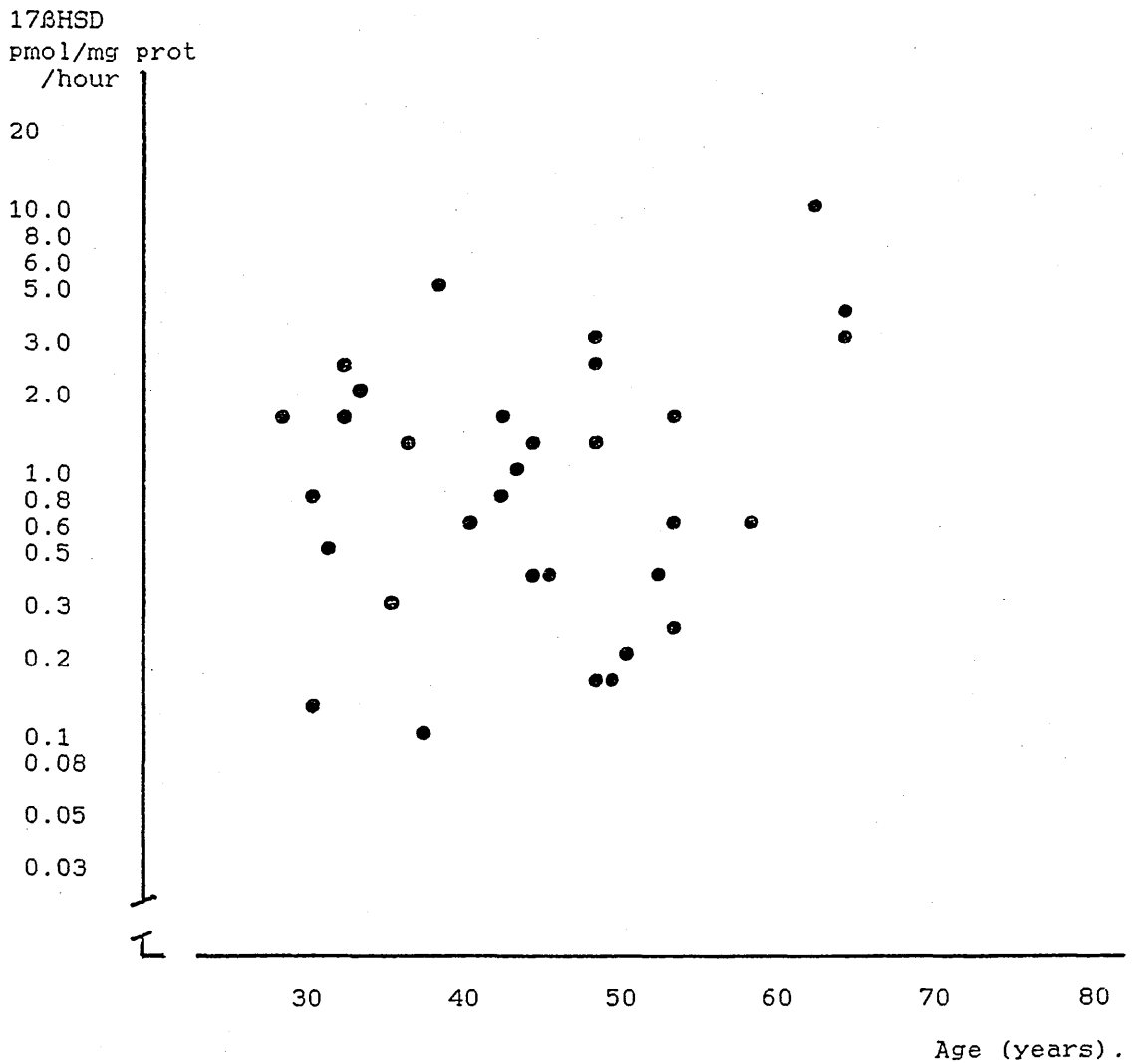



Figure 6:7b.

Adipose tissue 17ßHSD activity and patient age (benign disease).

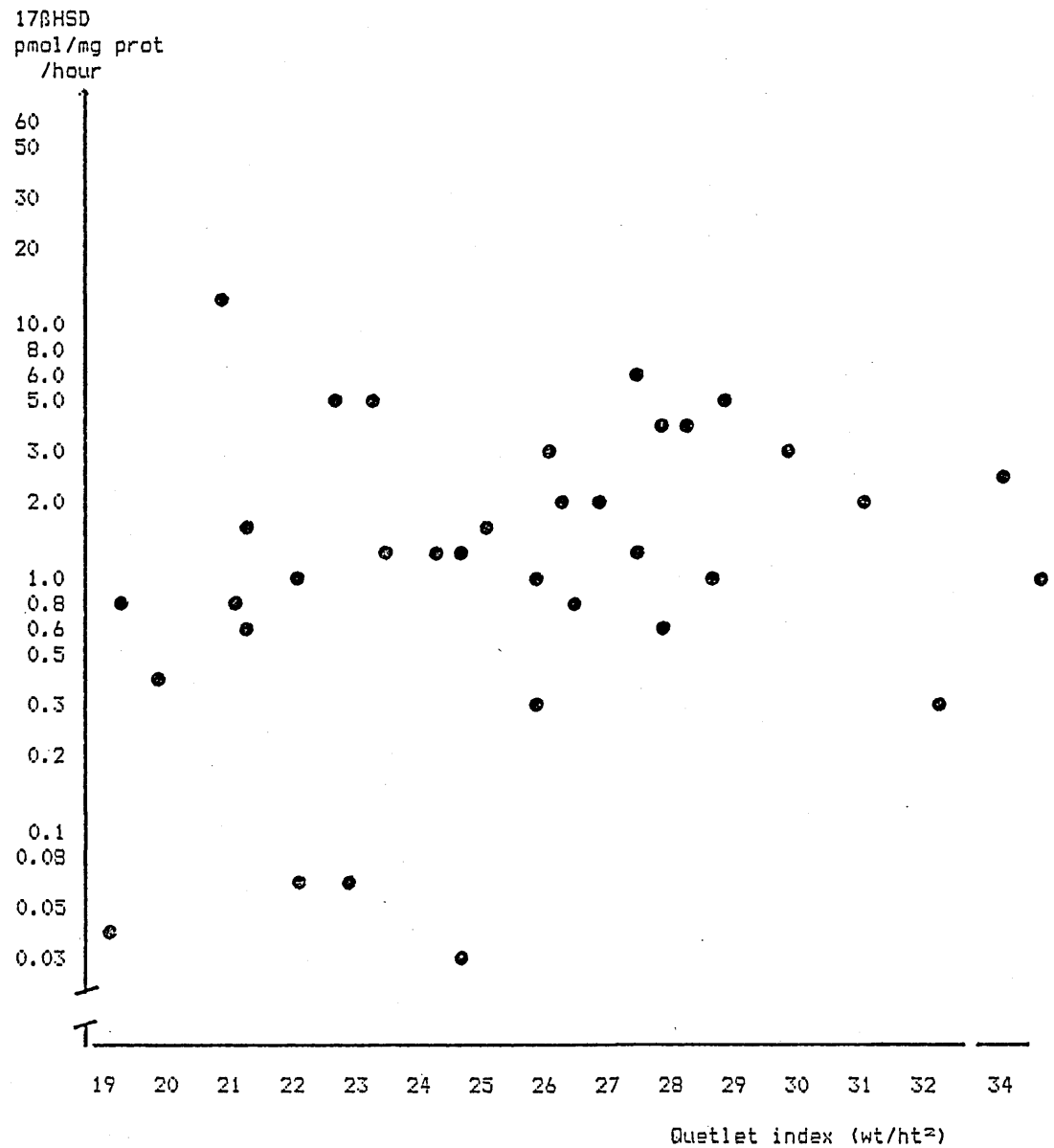



Figure 6:8a.

Adipose tissue 17ßHSD activity and obesity (cancer patients).

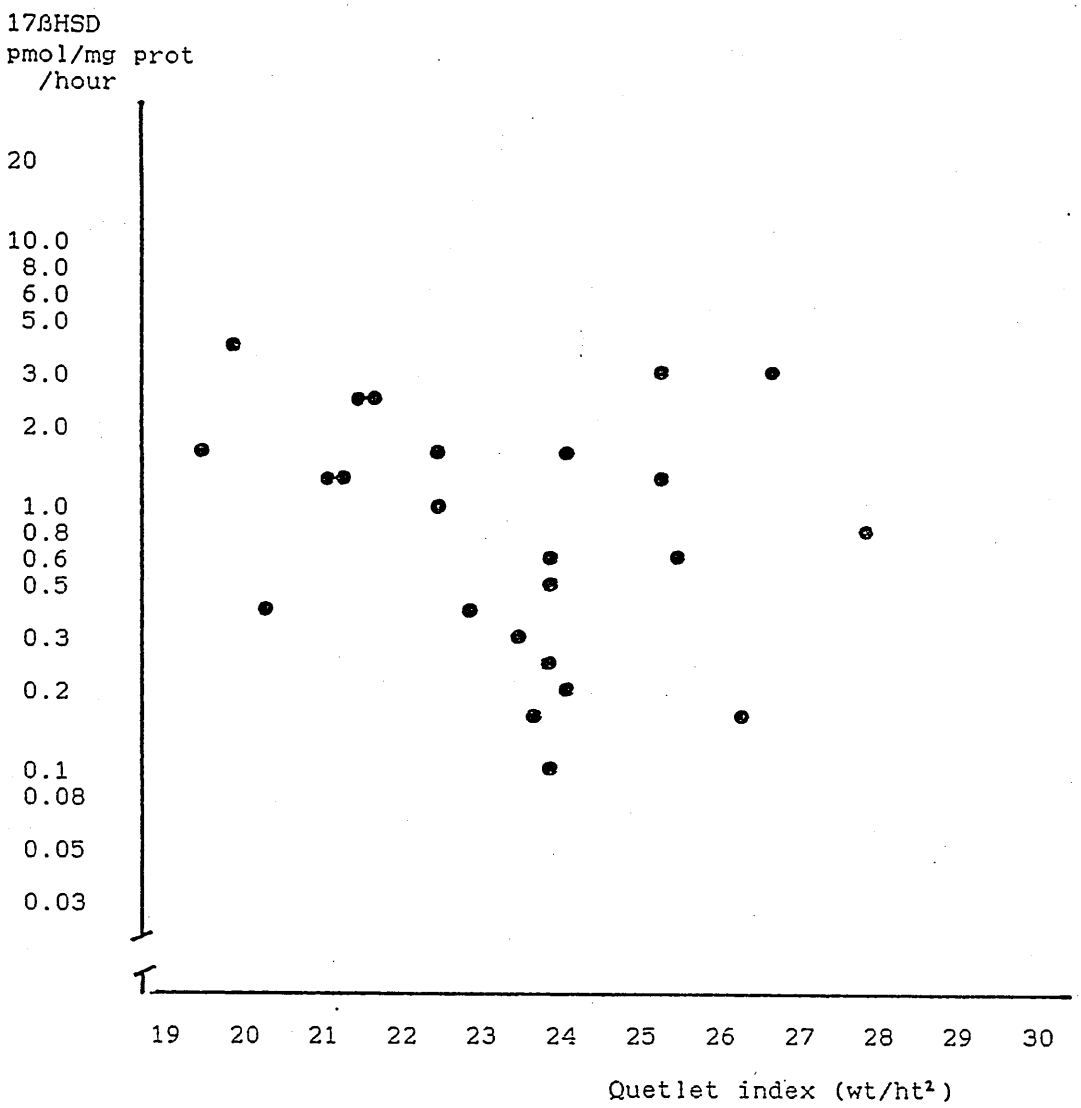



Figure 6:8b.

Adipose tissue 17ßHSD activity and obesity (benign disease).

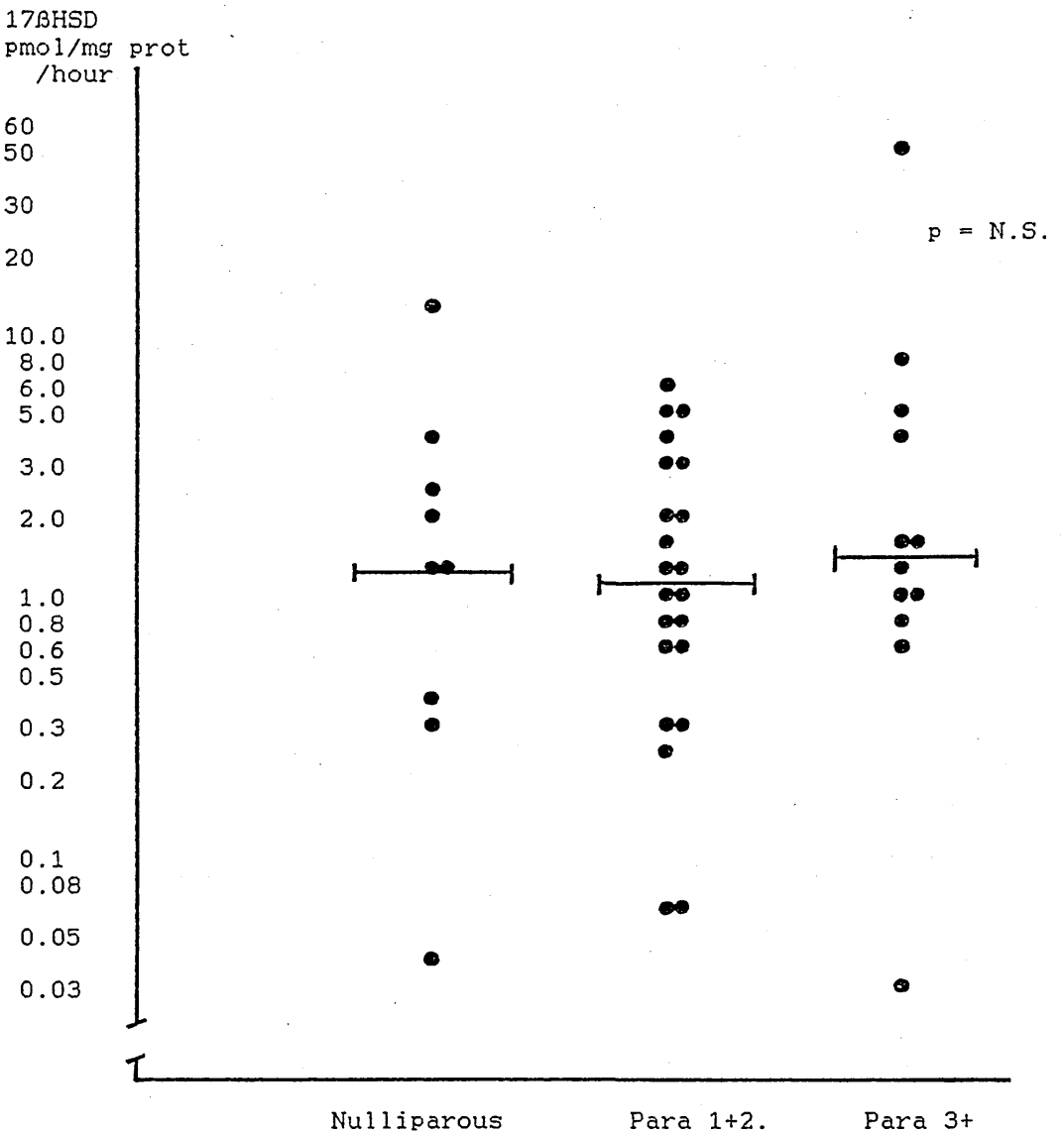



Figure 6:9a.

Adipose tissue 17ßHSD activity and parity (cancer patients).

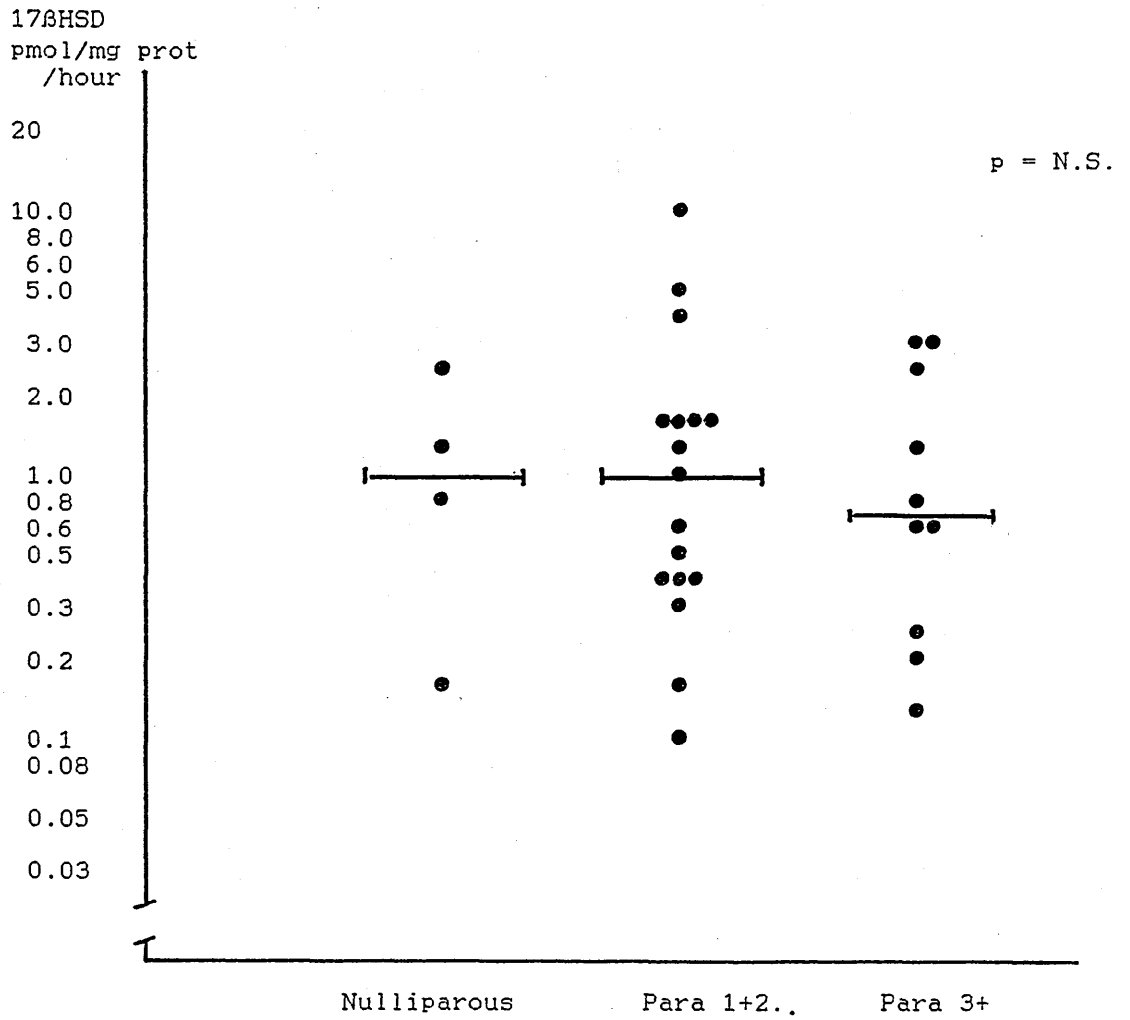



Figure 6:9b.

Adipose tissue 17ßHSD activity and parity (benign disease).

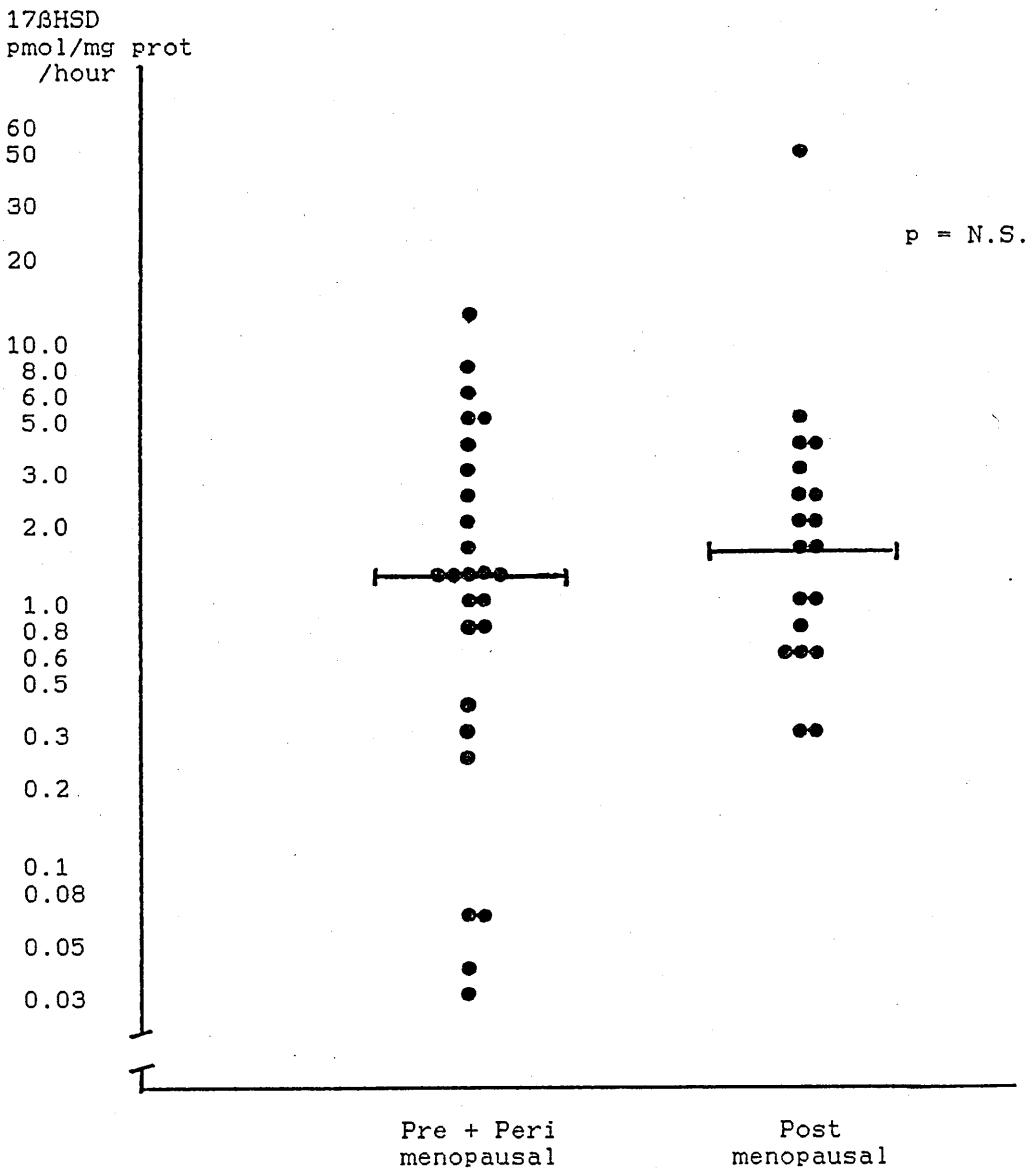



Figure 6:10a.

Adipose tissue 17ßHSD activity and menopausal status (cancer patients).

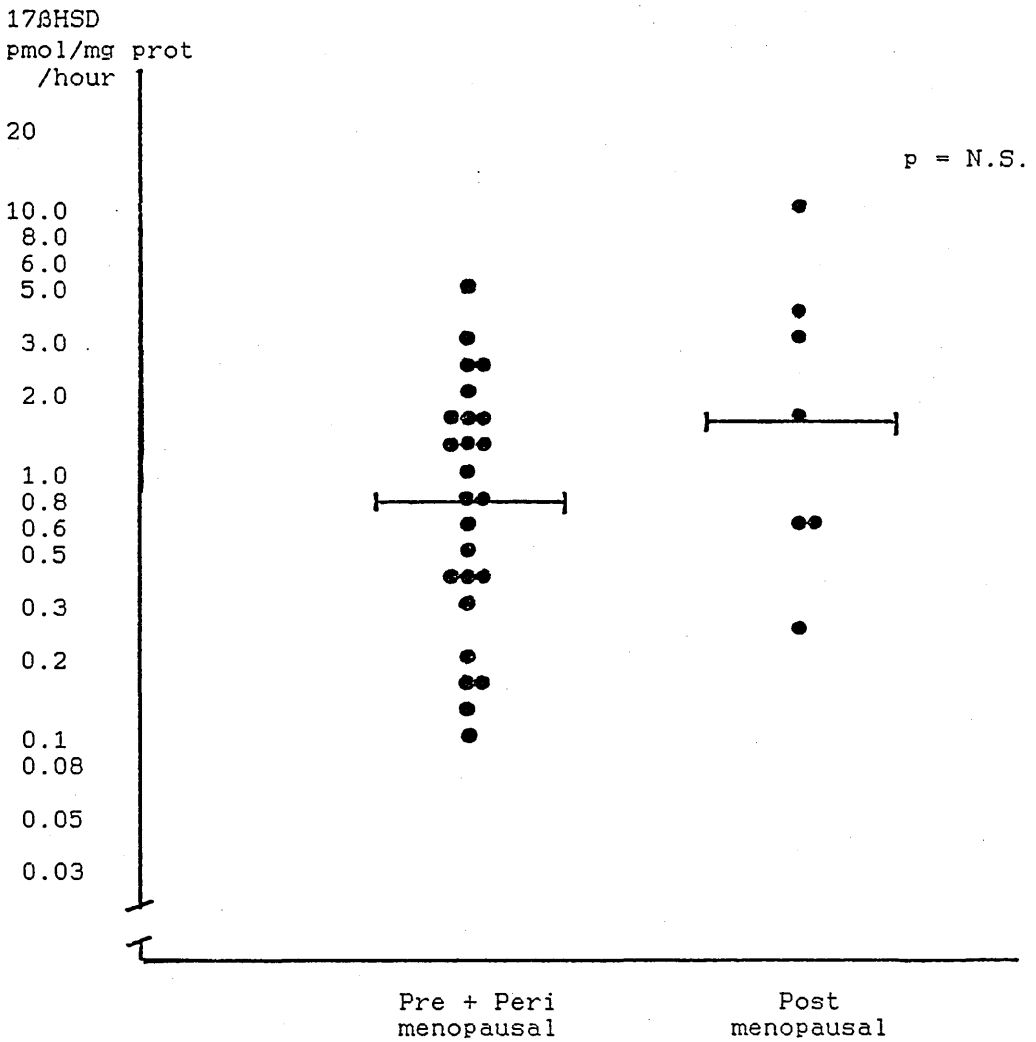



Figure 6:10b.

Adipose tissue 17ßHSD activity and menopausal status (benign disease).

## Discussion

This study confirms that all samples of breast adipose tissue possess 17BHSD activity with levels varying markedly between individuals. Levels of activity are generally higher than levels of aromatase in the same specimens (in only one case was the conversion of androstenedione to testosterone lower than the conversion to oestrogen). This finding and the levels of activity observed in these specimens are consistent with the results of other studies[10,121,123].

The fact that there is no significant difference in levels of activity between the breast cancer patients and those with benign disease is also in keeping with the earlier report of Beranek et al[10].

Levels of activity also vary between the quadrants of the twelve breasts studied, but with no consistent pattern of activity between the quadrants or in relation to the location of tumours in the breasts. In contrast to the distribution of aromatase activity higher activity is not associated with the upper outer quadrant (in seven cases activity was greater in the lower inner quadrant than in the upper outer quadrant). There is therefore no evidence from this study to support the suggestion by Beranek et al. that low levels of activity are found in the lower half of the breast[11]. This suggestion, however, was based on results from only four samples from lower breast quadrants with no samples being obtained from other quadrants of the same breast for comparison.

In contrast with the earlier results for aromatase activity, 17BHSD is not elevated in cancerous breasts or in tumour-bearing quadrants. It therefore seems unlikely that there is a non-specific increase in steroid metabolism around breast cancers.

The association between levels of 17BHSD activity and tumour size is interesting and has been observed previously[11]. A possible explanation for this phenomenon is that tumours can produce factors which stimulate 17BHSD activity in their surrounding tissues and that larger tumours would be expected to produce more of an effect. However in the majority of the mastectomy specimens studied higher levels of activity were detected in areas remote from the tumours. While this does not preclude an effect of tumours on enzyme activity, there must be other factors influencing enzyme activity in the breast. The association of elevated enzyme activity with large tumours may therefore not be entirely due to enhancement of local enzyme activity by these tumours.

It is possible, however, that locally enhanced enzyme activity could stimulate tumour growth by producing a local increase in the concentration of oestradiol. In keeping with this suggestion a number of studies have reported elevated concentrations of oestradiol in tumours and their surrounding tissues[14,51,99]. These two explanations are not mutually exclusive and it is possible that some tumours may stimulate their own growth by increasing levels of 17BHSD in their immediate surroundings in order to increase the local concentration of growth promoting hormones.

The fact that higher levels of activity are also seen around

tumours that have metastasised to regional lymph nodes is consistent with elevated enzyme activity being associated with more rapidly growing (and disseminating) tumours.

The present study fails to show any correlation between levels of 17BHSD and parity, menopausal status, weight or obesity. Although there have been no reports on the 17-beta hydroxy reduction of androstenedione being influenced by any of these factors, Beranek et al found that the rate of oestrone 17-beta hydroxy reduction was positively correlated with body weight[9] and Deslypere et al suggested that the oxidation of oestradiol was greater in premenopausal women[37]. Other studies, however fail to confirm these associations[121-124].

In conclusion, therefore there is no evidence for a non-specific increase in aromatase and 17BHSD activity around breast cancers. Levels of 17BHSD activity in adipose tissue however, appear to be related to tumour size and the presence of lymph node metastases. The possibility that tumours can influence levels of specific enzymes in their surrounding tissues cannot be excluded.

# Chapter 7.

Enzyme activity in adipose tissue immediately adjacent to tumours.

## Introduction

If the relationships between enzyme activities and breast cancer seen in the earlier studies reflects specific tumour enhancement of enzyme activity in their surrounding tissues then a gradient of activity should be detectable in surrounding tissues with higher activity in the immediate vicinity of the tumour. The aim of this study was therefore to investigate aromatase and 17BHSD activity in tissue from the immediate vicinity of tumours and compare these with the activities in samples from the same breast quadrant but which are more remote from the tumour.

## Methods:

Adipose tissue samples were obtained from the immediate vicinity of 9 tumours and also from within the same quadrant but more remote from the tumour. In a further case two tumours were present in the same breast. Samples were therefore obtained from the vicinity of both of these tumours and from an intermediate area of the breast. These samples were then processed as before and incubated with 1-beta $^{3}H$ androstenedione to measure both aromatase and 17BHSD activity.

## Results:

The results of the aromatase assays are shown in Table 7:1. Aromatase activity was higher in the immediate vicinity of 6 of the tumours but lower in the other 5. The results of the 17BHSD assays are shown in Table 7:2. Activity was higher in the immediate vicinity of only 4 of the tumours, with lower activity in the remaining 7.

Table 7:1.

Aromatase activity in adipose tissue adjacent to and remote from tumours.

| No. | SITE | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | AROMATASE %CONV. | AROMATASE fmol/mg prot /hour |
|---|---|---|---|---|---|
| 1 | ADJ 1 | 1 | 1.1 | .019 | 11.5 |
| | ADJ 2 | 1.1 | 1.7 | .046 | 18.0 |
| | REM | 0.8 | 0.9 | .033 | 24.4 |
| 3 | ADJ | 1.1 | 1.2 | .132 | 70.4 |
| | REM | 1 | 1.1 | .033 | 20 |
| 6 | ADJ | 2 | 2.9 | .128 | 29.4 |
| | REM | 2 | 2.2 | .011 | 3.3 |
| 12 | ADJ | 2 | 2.0 | .033 | 11 |
| | REM | 2 | 1.8 | .021 | 7.8 |
| 14 | ADJ | 1.9 | 1.0 | .017 | 11.3 |
| | REM | 2 | 2.5 | .12 | 32 |
| 15 | ADJ | 1.3 | 3.7 | .099 | 17.8 |
| | REM | 2 | 6.0 | .315 | 35 |
| 32 | ADJ | 2 | 2.1 | .055 | 17.5 |
| | REM | 2 | 1.6 | .053 | 22.1 |
| 39 | ADJ | 2 | 1.9 | .044 | 15.4 |
| | REM | 1.2 | 3.4 | .064 | 12.6 |
| 46 | ADJ | 1.6 | 1.4 | .046 | 21.9 |
| | REM | 2 | 1.8 | .022 | 8.1 |
| 48 | ADJ | 1 | 2.3 | .070 | 20.3 |
| | REM | 1 | 0.7 | .038 | 36.2 |
| 49 | ADJ | 2 | 2.2 | .067 | 20.3 |
| | REM | 2 | 3.0 | .035 | 7.8 |

Table 7:2.

17BHSD activity in adipose tissue adjacent to and remote from tumours.

| No. | SITE | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | 17BHSD %CONV | 17BHSD pmol/mg prot /hour |
|---|---|---|---|---|---|
| 1 | ADJ 1 | 1 | 1.1 | 2.4 | 1.44 |
| | ADJ 2 | 1.1 | 1.7 | 9.1 | 3.56 |
| | REM | 0.8 | 0.9 | 2.63 | 1.95 |
| 3 | ADJ | 1.1 | 1.2 | 3.93 | 2.1 |
| | REM | 1 | 1.1 | 1.35 | 0.8 |
| 6 | ADJ | 2 | 2.9 | 12.6 | 2.9 |
| | REM | 2 | 2.2 | 13.8 | 4.2 |
| 12 | ADJ | 2 | 2.0 | 4.34 | 1.4 |
| | REM | 2 | 1.8 | 2.18 | 0.8 |
| 14 | ADJ | 1.9 | 1.0 | 1.6 | 0.4 |
| | REM | 2 | 2.5 | 1.4 | 0.5 |
| 15 | ADJ | 1.3 | 3.7 | 3.0 | 0.5 |
| | REM | 2 | 6.0 | 14.9 | 1.7 |
| 39 | ADJ | 2 | 1.9 | 0.2 | 0.06 |
| | REM | 1.2 | 3.4 | 1.6 | 0.3 |
| 46 | ADJ | 1.6 | 1.4 | 16.4 | 7.8 |
| | REM | 2 | 1.8 | 24.3 | 9.0 |
| 48 | ADJ | 1 | 2.3 | 3.5 | 1.0 |
| | REM | 1 | 0.7 | 2.4 | 2.3 |
| 49 | ADJ | 2 | 2.2 | 0.86 | 0.26 |
| | REM | 2 | 3.0 | 0.92 | 0.20 |

Analysis of the results in relation to tumour type fails to indicate that certain tumour types are associated with enhanced enzyme activity. In addition there does not appear to be any correlation between the results for the two different enzymes.

Conclusion:

These results again highlight the wide variations in enzyme activity that occur within the breast. There is however no evidence for a zone of high aromatase or 17BHSD activity immediately around all tumours. It therefore seems unlikely that the earlier association of aromatase activity with breast cancer is entirely due to tumour enhancement of activity. The possibility still remains that some tumours may be capable of stimulating activity in their surroundings while others may be capable of inhibiting activity.

# Chapter 8.

## Steroid metabolism in breast adipose tissue of treated breast cancer patients.

## Introduction

The results presented in the earlier chapters suggest that steroid metabolism in breast adipose tissue may be involved in the evolution and continued growth of breast cancers. It is therefore possible that such local metabolism might influence the response of a cancer to systemic treatment. The purpose of this study was therefore to determine if levels of enzyme activity in breast adipose tissue are influenced by systemic therapy or correlate with the response to treatment.

## Methods

Twenty-two of the breast cancer patients included in the earlier studies had received either local or systemic treatment prior to mastectomy. Details of the treatments given are outlined in Table 8:1.

In seventeen patients treatment was administered prior to surgery in an attempt to reduce tumour size and determine the sensitivity of the primary tumours to systemic therapy. Details of patient selection and the modes of therapy are described elsewhere[58]. Seven patients received tamoxifen, three aminoglutethimide with hydrocortisone and three had an oophorectomy. Four of these who failed to respond to such endocrine therapy (oophorectomy n = 2; Tamoxifen n = 1; Aminoglutethimide n = 1) went on to have chemotherapy. A further four patients had chemotherapy alone. Treatment continued for three months and clinical response was monitored as accurately as possible by mammography and clinical examination. After treatment a mastectomy was performed. Response was considered complete if there was clinical resolution of the

Table 8:1.

Details of patients who received local or systemic therapy prior to mastectomy. Tumour size = pathologist's measurement. Size in brackets is the initial clinical measurement.

| Patient No. | Type of Carcinoma | Treatment | Tumour Size (cm) |
|---|---|---|---|
| 2 | No special type | Tam. > PR | 2.0 (4.5) |
| 9 | Lobular | Tam. no response | 6.0 |
| 10 | Ductal | oox. no response, CHOP > PR | 2.0 (5.8) |
| 12 | Mucoid | WLE + R/TH > recurrence | 1.5 |
| 14 | Adenocarcinoma | AMG > PR | 2.6 (5.2) |
| 16 | Mucoid | Oox > PR | 2.0 (5.0) |
| 17 | Ductal | Tam > PR | 2.5 (4.0) |
| 18 | Lobular | CHOP (defaulted) | 4.0 (5.2) |
| 22 | No special type | CHOP + R/TH + boost | 5.0 |
| 23 | Ductal | Tam > progression | 4.5 (3.2) |
| 26 | Adenocarcinoma | CHOP > CR | 0 (10.0) |
| 28 | No special type | CHOP > CR | 0 (3.0) |
| 30 | Ductal | AMG >Prog. Dis.>CHOP >CR | 0 (4.2) |
| 31 | Ductal | Tam >Prog. Dis.>CHOP >PR | 2.0 (4.5) |
| 33 | No special type | R/TH for in situ carcinoma | 5.0 |
| 36 | No special type | CHOP > PR | 1.5 (6.2) |
| 40 | Ductal | AMG (stopped - reaction) | 5.0 |
| 42 | Ductal | AMG > PR | 2.0 (5.2) |
| 43 | Ductal | Tam > PR | 1.8 (4.3) |
| 45 | Atypical Medullary | Oox >no response >CHOP >PR | 3.0 (5.2) |
| 47 | Spindle Cell | CHOP > no response | 3.7 |
| 51 | Ductal | WLE + CMF > local recurrence | 3.8 |

Tam. - Tamoxifen; Oox - oophorectomy; AMG - aminoglutethimide;
CHOP - Cyclophosphamide, doxorubicin, vincristine + prednisolone;
R/TH - Radiotherapy; WLE - wide local excision of breast cancer;
CMF - Cyclophosphamide, methotrexate + 5 fluorouracil;
CR - complete response; PR - partial response

tumour with no histologically detectable tumour at mastectomy. A partial response was indicated by a reduction in tumour size, with tumour still detectable at mastectomy. Absence of tumour regression indicated a failure of response. Samples of breast adipose tissue were obtained at the time of mastectomy (if complete tumour regression had occured, tissue was taken from the area around the site of the resolved tumour).

Adipose tissue was also obtained from one patient (No. 18) who failed to complete a full course of chemotherapy and another (No. 40) who had to discontinue treatment with aminoglutethimide because of an adverse reaction. These patients could therefore not be adequately assessed for response.

Tissue samples were obtained at the time of mastectomy from a further three patients who developed local recurrence following wide local excision of small tumours. Two of these patients (Nos. 12,33) had received post-operative radiotherapy, the other (No. 51) received adjuvant chemotherapy. Tissue was also obtained from a patient (No. 22) who presented with a locally advanced tumour (T4). This tumour responded to a combination of chemotherapy and radiotherapy and was then treated by mastectomy.

In all patients, adipose tissue was obtained at the time of mastectomy, i.e. after local or systemic therapy. Assays of aromatase and 17BHSD activity in the adipose tissue samples from these patients were performed as part of the earlier experiments (Chapters 3 & 6). In one case (No. 2) aromatase activity alone was measured.

Results:

The results of the aromatase and 17BHSD assays on breast adipose tissue from these patients are shown in Table 8:2. Aromatase and 17BHSD activities are subdivided according to the type of therapy in Figures 8:1 and 8:2. The median levels of activity for each enzyme are indicated on the figures. Because of the small number of patients in each subgroup, valid statistical analysis is not feasible. Certain observations can however be made.

All three patients who were treated by oophorectomy have aromatase activity which is higher than the median. In contrast only one of the six patients treated with tamoxifen had aromatase activity greater than the median. It is therefore possible that oophorectomy may enhance peripheral aromatase activity while tamoxifen may inhibit activity. Neither aminoglutethimide nor chemotherapy appear to be associated with particularly high or low aromatase activity. None of the therapies are associated with either high or low 17BHSD activity.

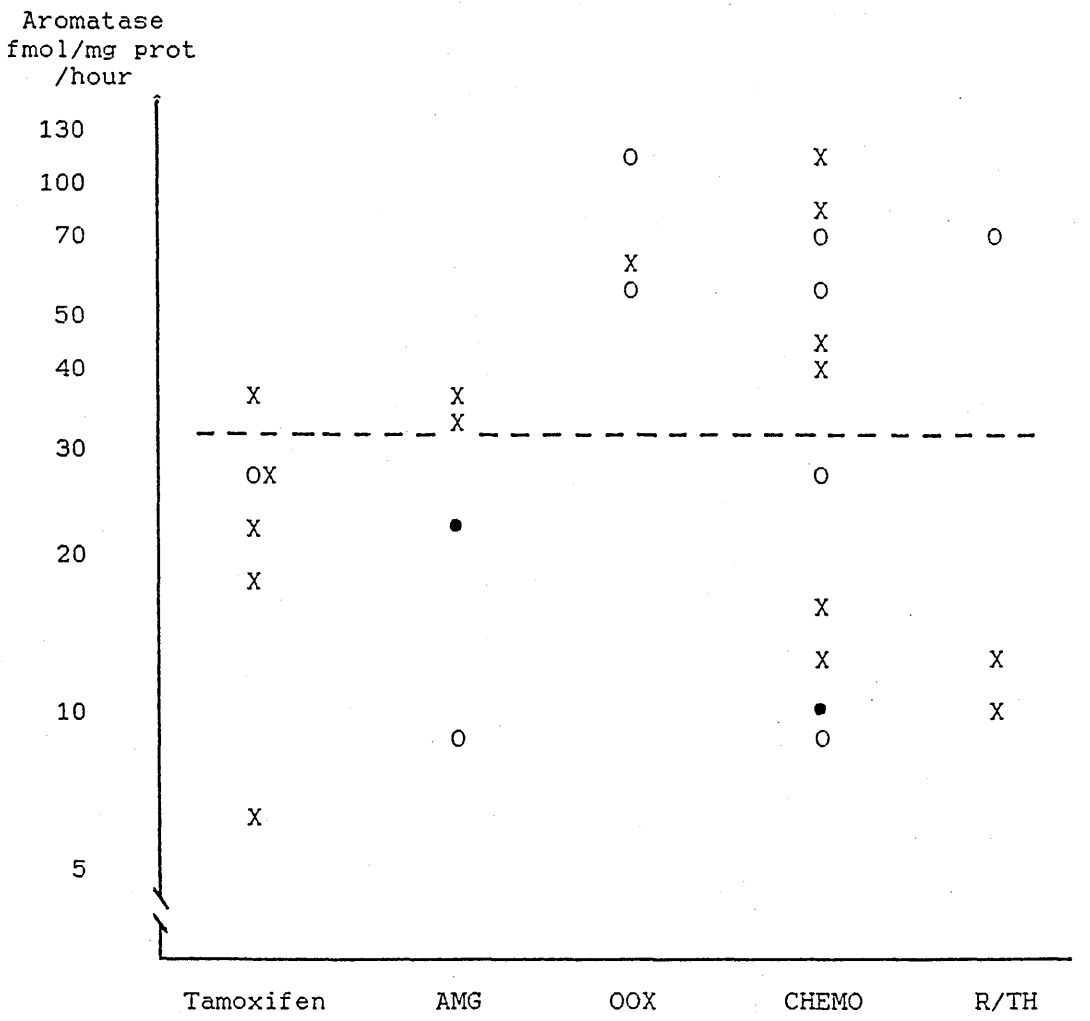
Activity of both enzymes is low in the two patients who received adjuvant radiotherapy, whereas there is high activity of both enzymes in the patient who received therapeutic radiotherapy. Radiotherapy is known to affect tissues by producing an initial inflammatory response with an increase in cellularity. Resolution of these changes results in decreased cellularity with an increase in fibrous tissue. Although no firm conclusion can be drawn from only three patients, the observed results are consistent with

Table 8:2.

Assay results for patients who received systemic therapy prior to surgery.

| No. | Treatment | AROMATASE %conv. | AROMATASE fmol/mg /hour | 17BHSD %conv | 17BHSD pmol/mg /hour |
|---|---|---|---|---|---|
| 2 | Tam. > resp | .073 | 27 | - | - |
| 9 | Tam. no resp | .067 | 34.9 | 7.0 | 3.6 |
| 10 | oox. no resp, CHOP > resp | .179 | 114 | 1.4 | 0.9 |
| 12 | WLE + R/TH > Mx | .033 | 11 | 4.3 | 1.4 |
| 14 | AMG > resp | .12 | 32 | 1.4 | 0.5 |
| 16 | Oox > resp | .163 | 63.5 | 2.1 | 0.8 |
| 17 | Tam > resp | .075 | 18.5 | 4.1 | 1.0 |
| 18 | CHOP (defaulted) > Mx | .021 | 10.8 | 1.8 | 0.9 |
| 22 | CHOP + R/TH + boost > Mx + Cl | .066 | 78.6 | 38 | 52.8 |
| 23 | Tam > progression | .078 | 23.7 | 13.8 | 4.1 |
| 26 | CHOP > Comp. Resp | .039 | 14 | 0.18 | 0.06 |
| 28 | CHOP > Comp. Resp | .165 | 44 | 3.0 | 0.8 |
| 30 | AMG >Prog. Dis.>CHOP >Comp R | .036 | 9.2 | 1.2 | 0.3 |
| 31 | Tam >Prog. Dis.>CHOP >PR | .046 | 26.9 | 1.1 | 0.8 |
| 33 | R/TH for in situ > recur > Mx | .050 | 14 | 0.16 | 0.04 |
| 36 | CHOP > resp | .08 | 88.8 | 10 | 11.1 |
| 40 | Mx + Cl (AMG stopped) | .024 | 22.2 | 3.4 | 3.3 |
| 42 | AMG > resp | .047 | 36.4 | 1.7 | 1.3 |
| 43 | Tam > resp | .015 | 6.7 | 1.0 | 0.6 |
| 45 | Oox >no resp >Chop >resp | .272 | 56.7 | 9.0 | 1.9 |
| 47 | CHOP > no resp | .122 | 17.3 | 19.7 | 6.55 |
| 51 | Cons surg CMF > local recur. | .093 | 41.3 | 9.2 | 4.1 |

Abbreviations: see Table 8:1.

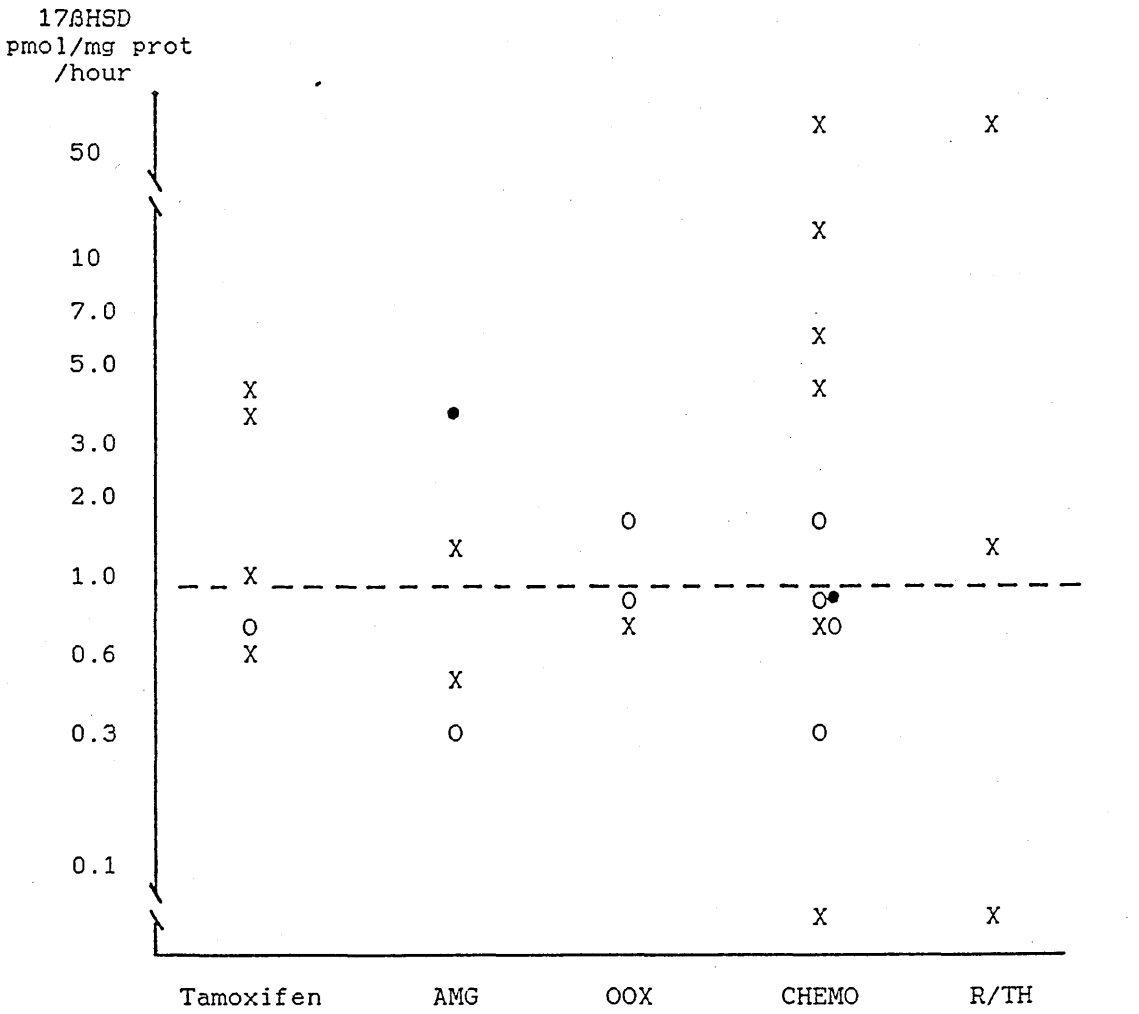



X - single therapy O - hormonal + chemotherapy • - incomplete therapy
AMG = aminoglutethimide + hydrocortisone; OOX = oophorectomy
CHEMO = chemotherapy; R/TH = radiotherapy

Figure 8:1.

Aromatase activity in breast adipose tissue of previously treated patients.

The broken line indicates the median level of activity for this group of patients.




X - single therapy O - hormonal + chemotherapy • - incomplete therapy
AMG = aminoglutethimide + hydrocortisone; OOX = oophorectomy
CHEMO = chemotherapy; R/TH = radiotherapy

Figure 8:2.

17ßHSD activity in breast adipose tissue and previous therapy

The broken line indicates the median level of activity for this group of patients.

radiotherapy influencing local metabolism by an effect on cellularity. In view of this possibility, and the fact that two of the patients received adjuvant radiotherapy and therefore could not be assessed for their initial response to treatment it was decided to exclude the irradiated patients from analysis of enzyme activity in relation to treatment response.

The two patients who did not complete courses of treatment were not assessed for response and were therefore excluded from further analysis.

Of the seventeen remaining patients there were three complete responses and ten partial responses. Four patients failed to respond. The four patients who received chemotherapy after failing to respond to hormonal manipulations all responded to chemotherapy. Tissue samples were only obtained from these patients after completion of chemotherapy. Details of the responses to treatment are outlined in Table 8:1. The relationship between aromatase activity and response is shown in Figure 8:3. Similar analysis of 17BHSD activity is shown in Figure 8:4. There is no apparent relationship between levels of aromatase activity and response to treatment. Significantly higher 17BHSD activity however, was found in non-responders (median 4.1 units) than in responders (median 0.8 units) [$p < 0.02$ by Wilcoxon rank test]. The lowest levels of activity were found in patients with complete responses (median 0.3 units). Only one of the twelve patients who responded to treatment had activity > 2.0 units, whereas the lowest activity detected in a non-responder was 3.6 units.

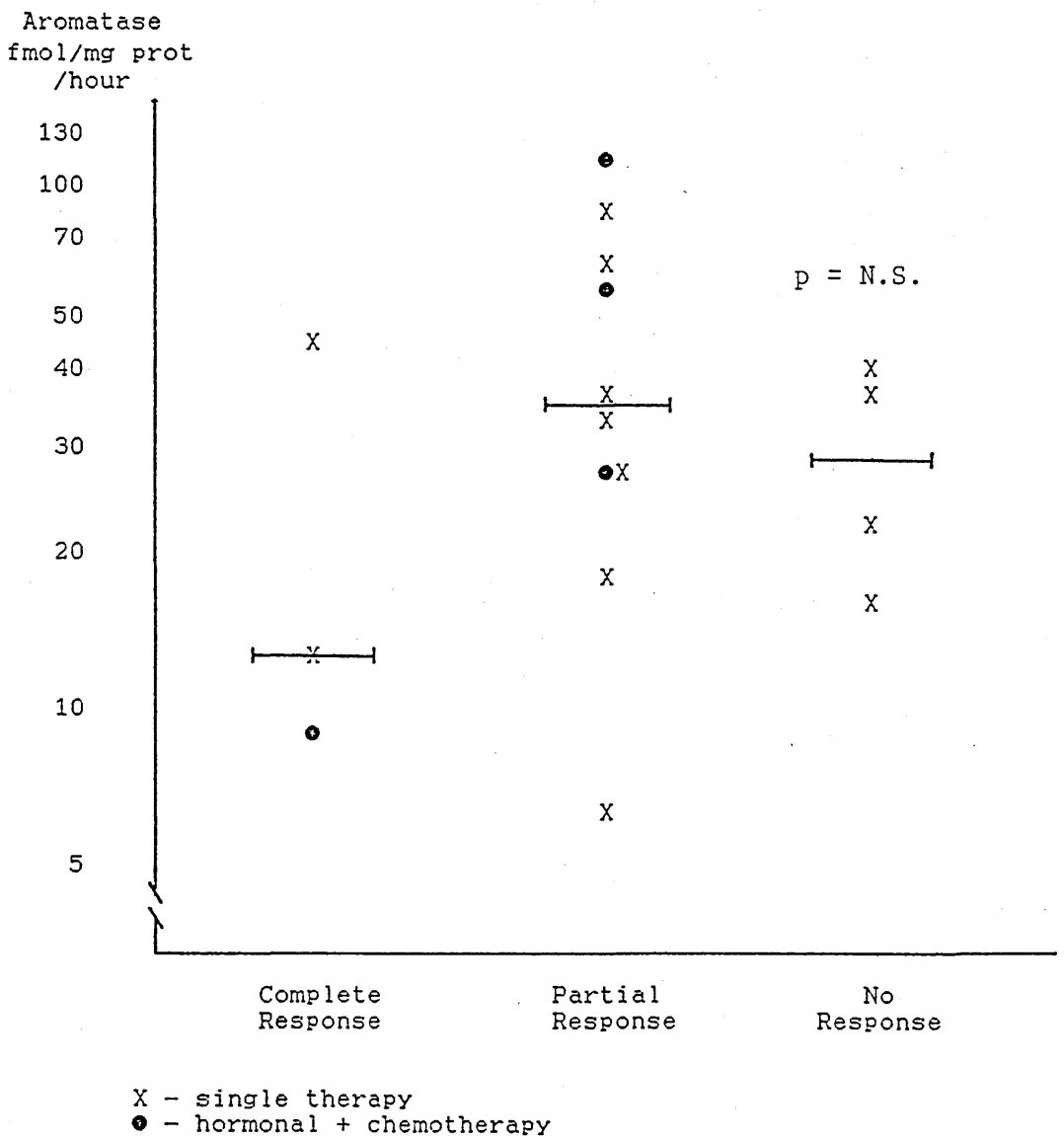



Figure 8:3.

Aromatase activity in breast adipose tissue and response to therapy.

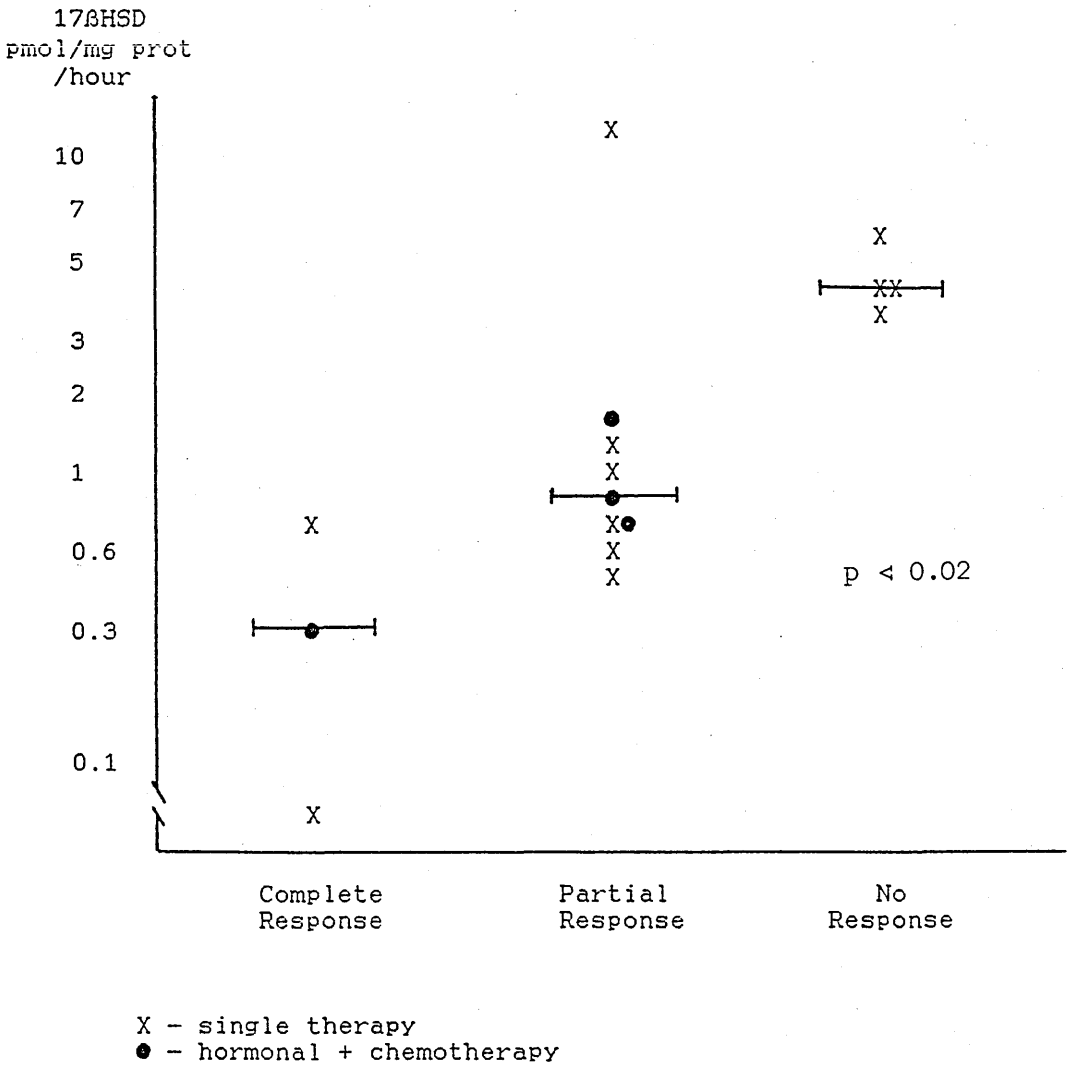



Figure 8:4.

17ßHSD activity in breast adipose tissue and response to therapy.

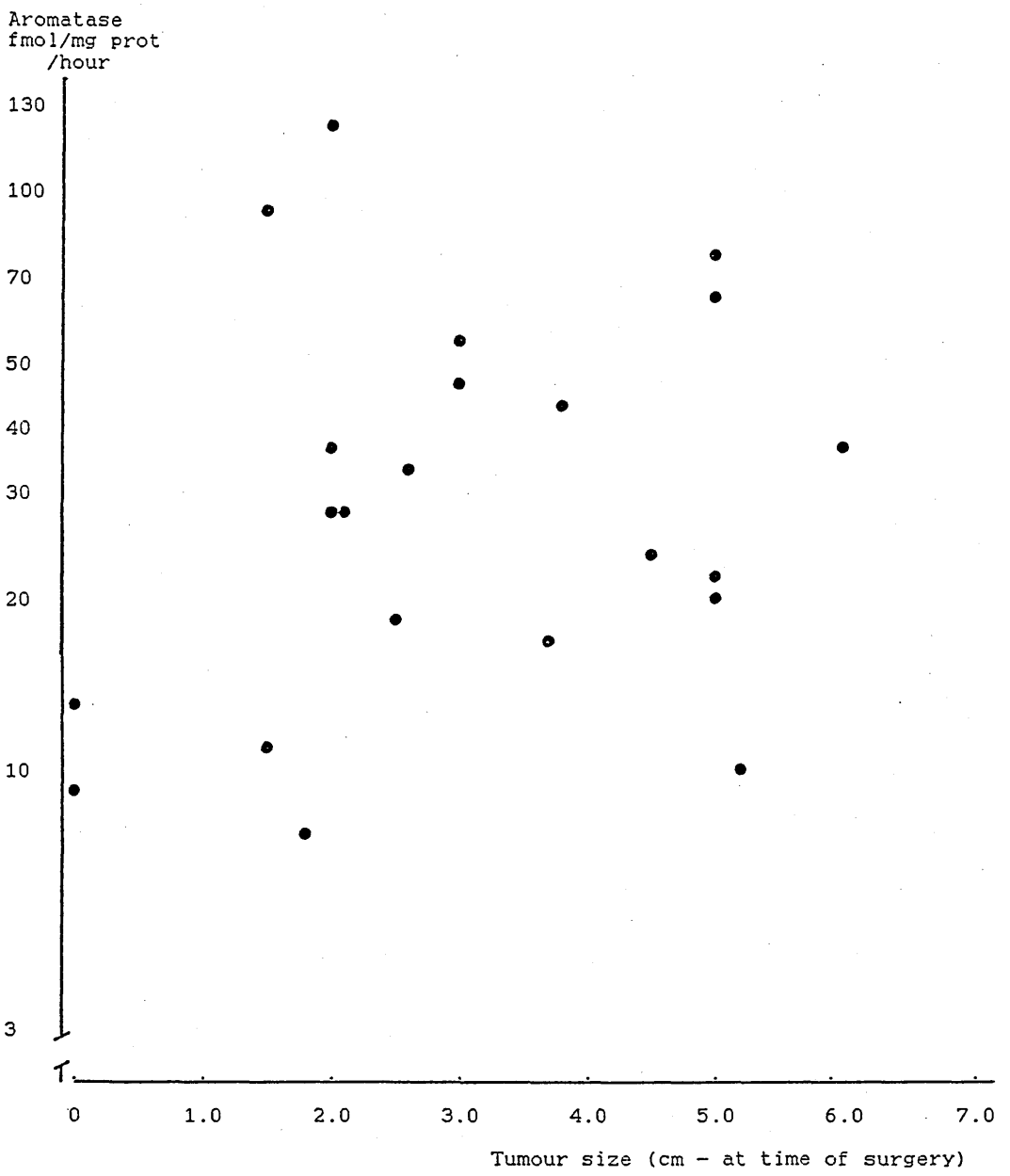



Figure 8:5.

Adipose tissue aromatase activity and tumour size (treated patients).

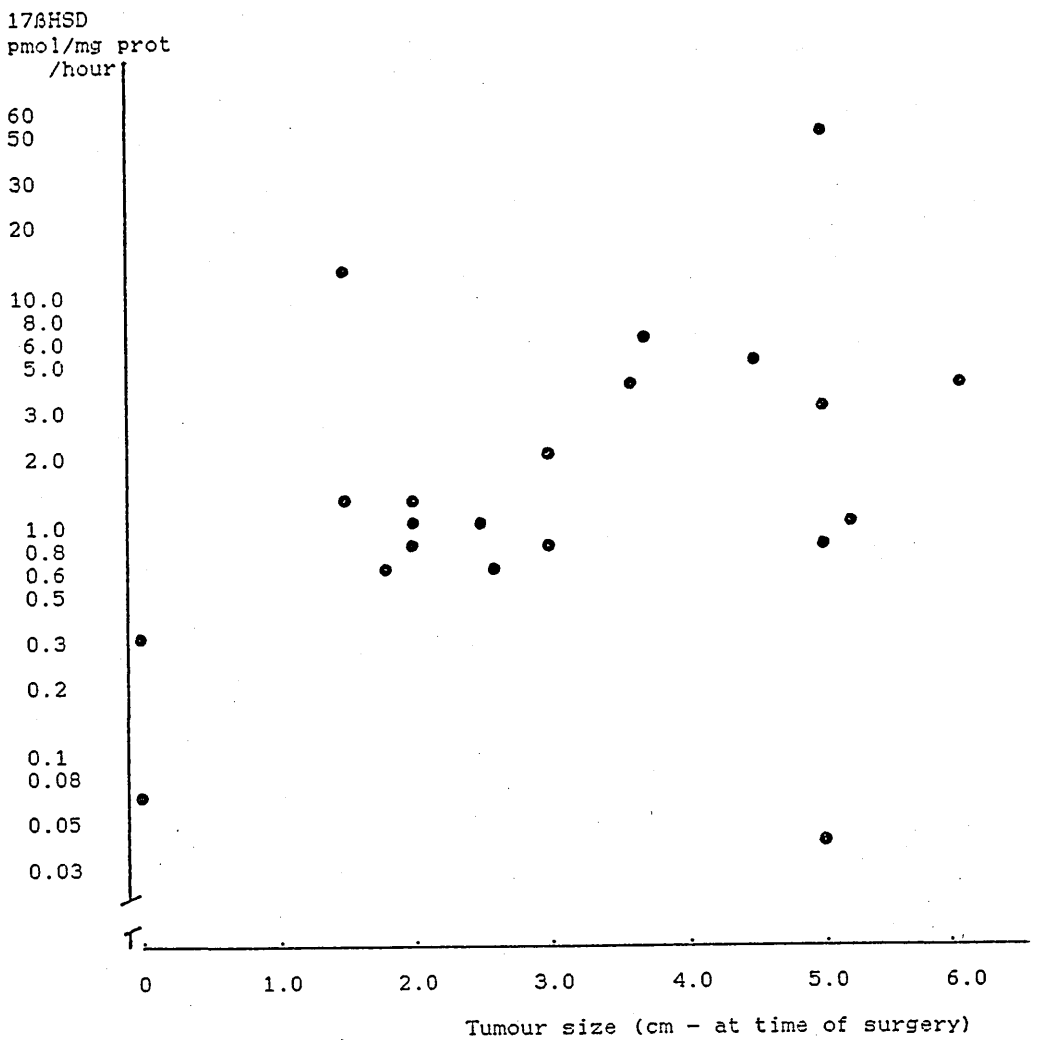



Figure 8:6.

Adipose tissue 17ßHSD activity and tumour size (treated patients).

Activity of both enzymes is plotted against tumour size in Figures 8:5 and 8:6. There is no statistically significant correlation between activity of either enzyme and tumour size. (aromatase vs size, r = -0.09, 17BHSD vs size, r = 0.31 p = NS).

Analysis of enzyme activity in relation to the responses to the individual treatments is again difficult because of the small numbers in each subgroup and the fact that four patients received both hormonal and chemotherapy. Figures 8:7-8:10 compare aromatase and 17BHSD activity with the responses to the various treatments used.

Response to tamoxifen, oophorectomy or chemotherapy appears to be associated with relatively low 17BHSD activity. The patients who responded to aminoglutethimide had higher levels of both aromatase and 17BHSD activity than the patient who failed to respond. Two of the three patients who responded to tamoxifen had lower levels of aromatase activity than the patients who failed to respond.

Discussion

Forrest[58] has recently suggested that the response of breast tumours to systemic treatment may be determined by the biochemical features of the tumour. There have been no previous reports suggesting that steroid metabolism in the tissues surrounding tumours may also be important.

With the variety of therapies used in these patients, only small numbers of patients received any one therapy. No firm conclusions can therefore be reached on the role of local steroid metabolism in

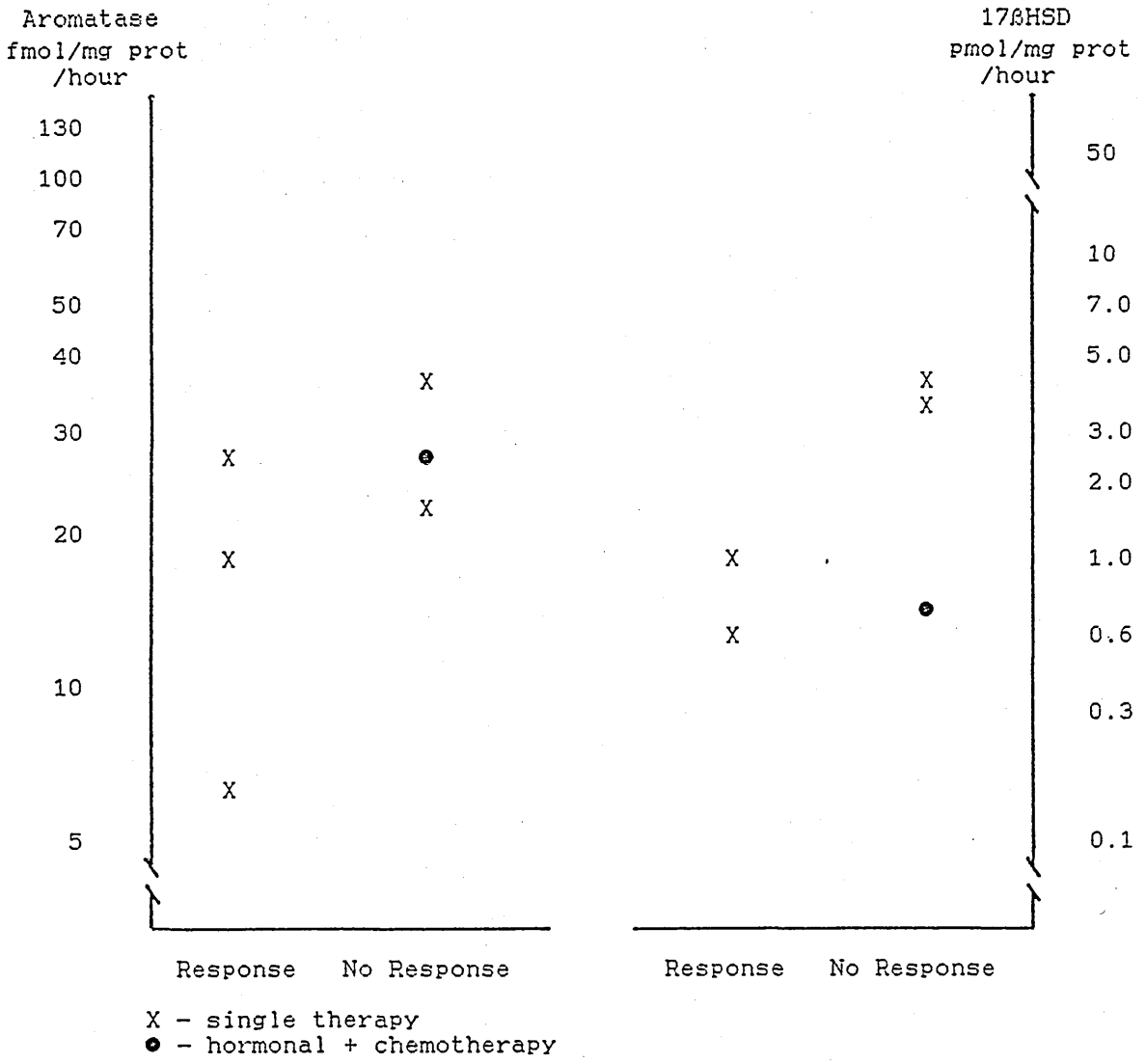



Figure 8:7.

Steroid metabolism in breast adipose tissue and response to tamoxifen.

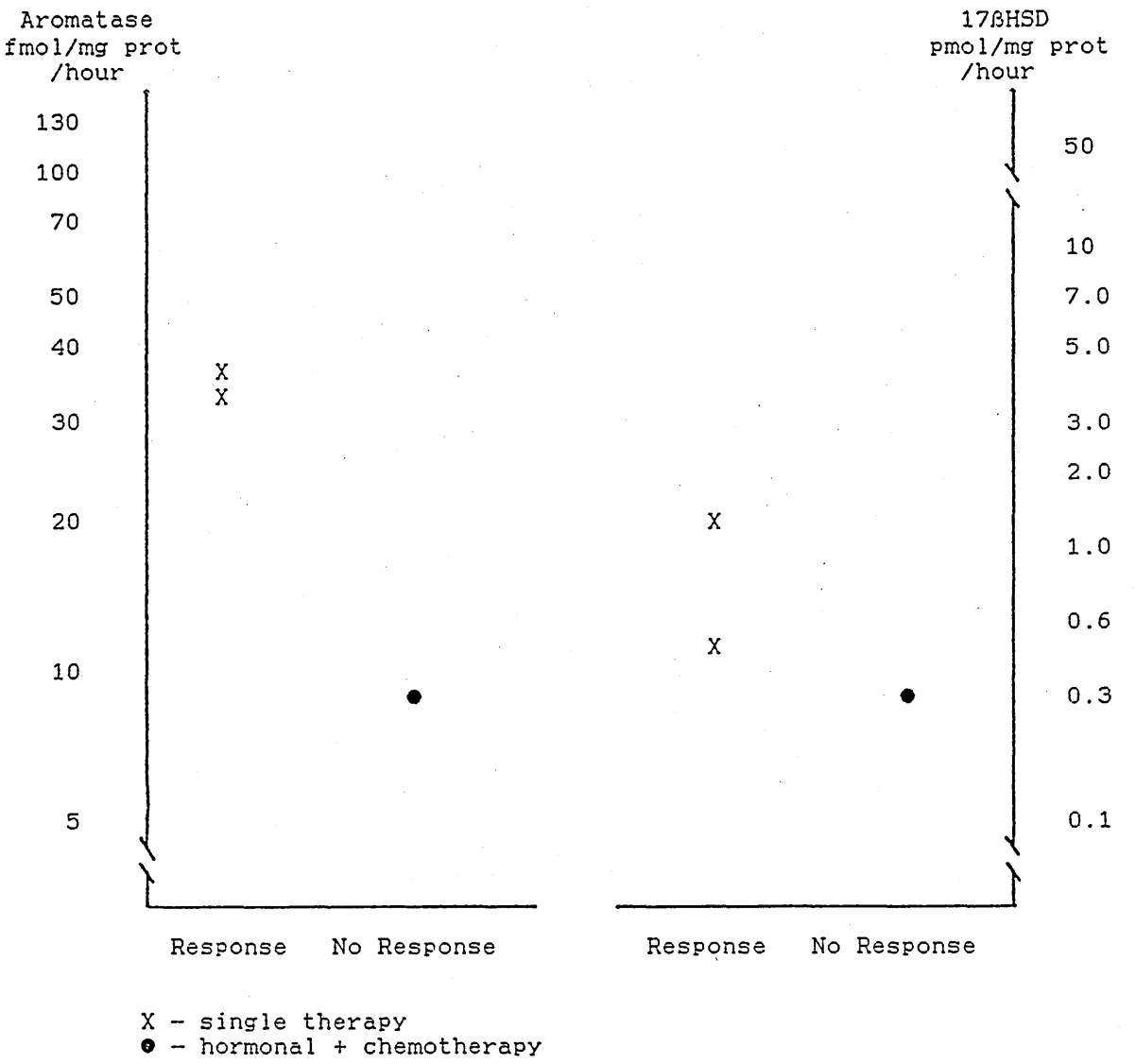



Figure 8:8.

Steroid metabolism in breast adipose tissue and response to aminoglutethimide.

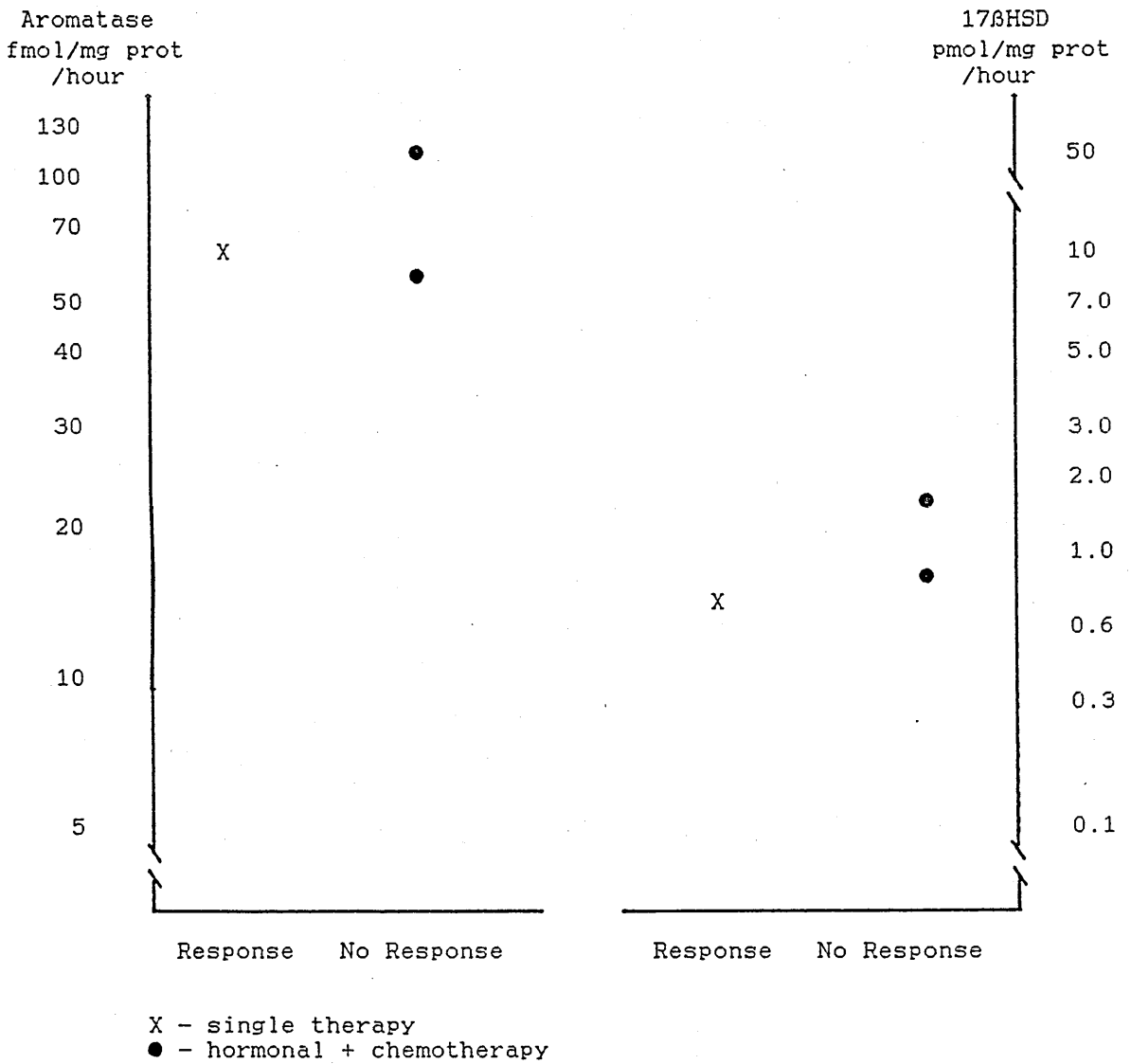



Figure 8:9.

Steroid metabolism in breast adipose tissue and response to oophorectomy.

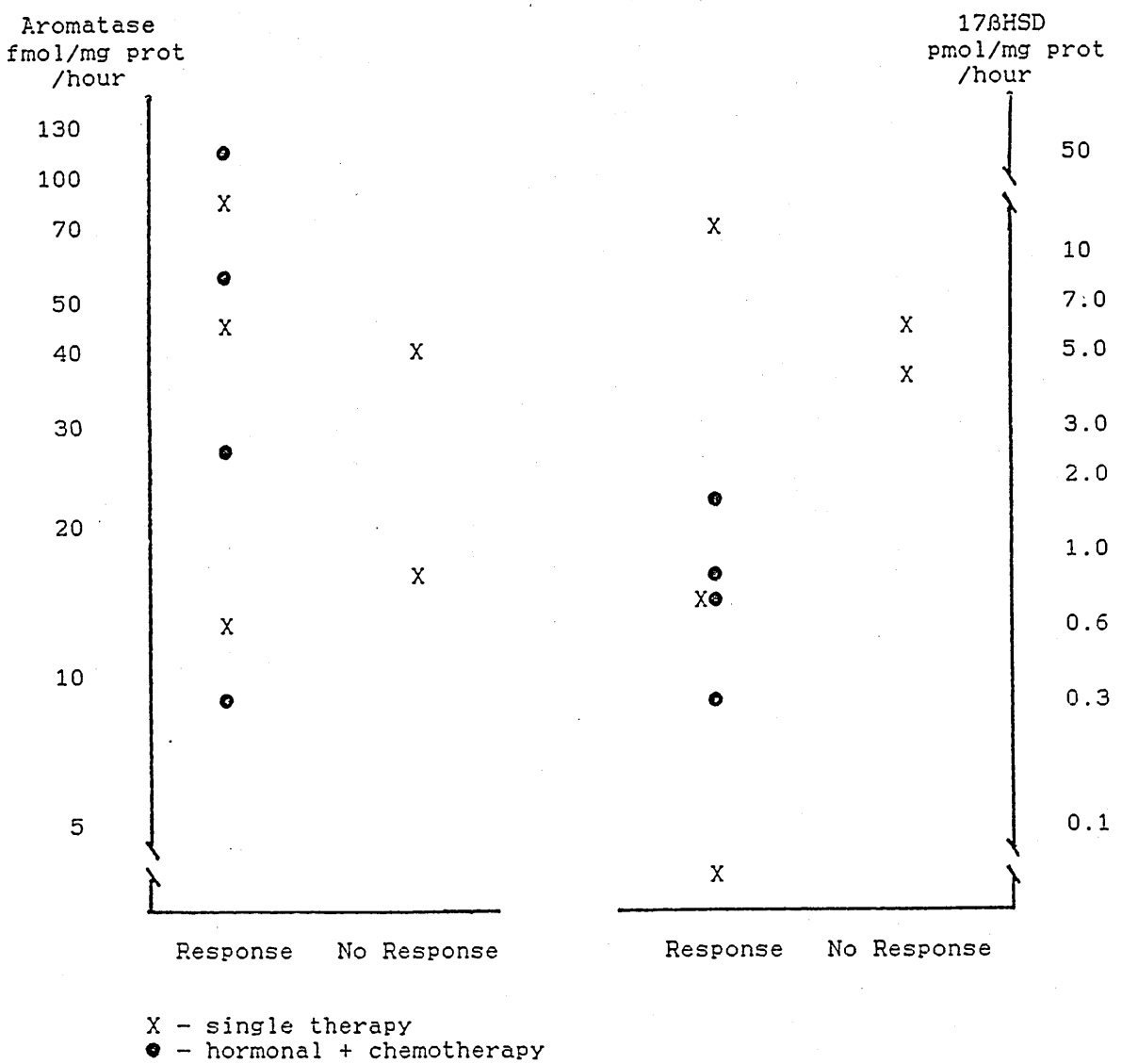



Figure 8:10.

Steroid metabolism in breast adipose tissue and response to chemotherapy.

the response to particular therapies. In addition, since samples of tissue were only obtained after treatment it is uncertain whether the treatment or the response of the tumours to treatment has influenced levels of activity. There are however, certain interesting trends.

With the possible exception of radiotherapy, there is no evidence to suggest that any of the treatments consistently enhance or inhibit peripheral 17BHSD activity. The results from the irradiated patients are consistent with radiotherapy initially stimulating activity but later leading to decreased activity.

Aromatase activity in adipose tissue is high in the three patients treated by oophorectomy. This is of interest in view of the earlier finding of high aromatase activity in perimenopausal women (Chapter 3). Folkerd[55] has also noted enhanced aromatase activity in adipose tissue from peri-menopausal women and has suggested that peripheral aromatase activity could be stimulated by high levels of FSH. If this is the case, then it must only be a temporary effect since there is no evidence from the earlier studies (Chapter 3) or those of others[10,37], to indicate that activity is higher in postmenopausal women, who have high levels of FSH.

The observation of relatively low aromatase activity in patients treated by tamoxifen with the lowest levels being seen in the patients who responded to this treatment is also interesting. Perhaps tamoxifen reduces local production of oestrogen in addition to preventing oestrogen from binding to the oestrogen receptor[153]. An alternative explanation is that anti-oestrogen

therapy is more likely to be effective in patients with reduced levels of local oestrogen production.

Aminoglutethimide is an aromatase inhibitor[78] and should therefore be effective against tumours which rely on oestrogen biosynthesis for their continued growth. It is therefore of interest that the patients who responded to aminoglutethimide had higher levels of aromatase activity than the patient who failed to respond. Whether such aromatase activity in adipose tissue is crucial for tumour growth or merely indicates the capacity of that patient's tissues to synthesize oestrogen remains to be determined. Another explanation, however, is that aminoglutethimide enhances peripheral aromatase activity. Such a phenomenon has been observed in breast tumours[107]. The fact that levels of aromatase activity in these patients were not higher than those observed in untreated patients makes such an explanation seem unconvincing.

The relationship between 17BHSD activity and response to all modes of treatment is striking. Higher levels of activity are associated with failure to respond, whereas lower levels are found in those who responded to therapy. There are a number of possible interpretations of this phenomenon. This could be a further manifestation of the relationship between 17BHSD activity and tumour size. Although the relationship between 17BHSD activity and tumour size at the time of surgery is not statistically significant, there is once again a trend for the highest levels of enzyme activity to be associated with the larger tumours. At the time of surgery the tumours which had responded to treatment were small and were associated with lower levels of 17BHSD than the larger tumours which failed to respond. Such an explanation implies

that local levels of 17BHSD are determined by the tumour; with levels of activity falling as tumours regress. If tumour growth is enhanced by locally elevated enzyme activity, these tumours, which were initally large, should have been associated with high enzyme activity. Investigation of levels of activity before and after treatment is necessary to determine whether levels of enzyme activity fall as tumours respond to therapy.

An alternative explanation is that enhanced local 17BHSD activity may be associated with tumours which are resistant to systemic therapy whereas low activity is found around tumours that will respond to therapy. If this is the case, then measurement of local enzyme activity could provide an important guide to the likely therapeutic response of a tumour.

While there are insufficient numbers of patients in the present study to permit firm conclusions on the role of local steroid metabolism in the progression or regression of breast tumours, the results are consistent with local steroid metabolism being involved in the response of tumours to therapy, implying that modification of local steroid metabolism could influence the outcome of the disease. Information on levels of enzyme activity before and after treatment in a larger series of patients should help to clarify the situation.

# Chapter 9.

Steroid metabolism and the fatty acid composition of breast adipose tissue.

Introduction

The foregoing studies, and those of others[10,14,37,55,57,115,121,122,123], have revealed that substantial individual variation occurs in levels of both aromatase and 17BHSD activity in breast adipose tissue. The factors which influence levels of activity and could account for such variation have been the subject of of considerable investigation[29,87,103]. In vitro experiments indicate that androgens[15,16,37], progestogens[12,68,129], epidermal growth factor and transforming growth factor alpha[101] can alter 17BHSD activity, and one report has shown that progesterone enhances activity in vivo[60]. Various substances, including corticosteroids, cyclic AMP, methyl xanthines and prolactin are known to influence aromatase activity in vitro[29,54,103,147,148], but to date there is little evidence that any of these factors operate in vivo.

It has been suggested that a high fat diet may increase oestrogen biosynthesis in adipose tissue[166]. Experiments have shown that changes in dietary fat can alter plasma hormone levels[79], and influence the menstrual cycle[80]. Since the composition of the diet determines the fatty acid composition of adipose tissue[48,75,116], it is not unreasonable to suggest that dietary fat could influence metabolism in adipose tissue.

Aim:

To measure the relative proportions of the fatty acids present in breast adipose tissue and to correlate levels with the aromatase and 17BHSD activity in these samples.

Methods

Tissue samples:

Stored samples of adipose tissue from 10 of the patients studied earlier were analysed for fatty acid content. Samples were chosen to include a wide range of enzyme activities and, in two cases, samples from different breast quadrants with differing enzyme activities.

Analysis of fatty acids.

(The analyses were kindly performed by Dr Rudolph A Riemersma, Senior Lecturer, Department of Cadiovascular Research, Edinburgh University.) Details of the methodology have been described elsewhere[168]. Lipids were extracted into redistilled heptane, and the extract was washed with isopropanol/0.05% potassium hydroxide by volume, to remove non-esterified fatty acids and phospholipids. The neutral lipid extract was reduced to dryness under vacuum then dissolved in dry toluene. Fatty acid methyl esters were prepared by direct transmethylation (10 min. at 50°C, with 0.5 mol/l sodium methoxide in methanol). The fatty-acid esters were washed with acidified water and re-extracted into hexane; the hexane layer was evaporated under vacuum. The methyl esters were redissolved in 40ul redistilled chloroform, ready for analysis on a Pye Chromatograph 204, fitted with a 1.5 m column, packed with GP 10% SP-2330 on 100/120 mesh 'Chromosorb WAW' (Supelco). The peaks were quantified with a flame ionisation detector and 'Tri-Vector III' integrator. The methyl-ester peaks in the chromatograms were identified by a combination of argentation thin layer chromatography and comparison of retention times with those of authentic fatty-acid methyl esters (Pufa 1 and 2 and NIH mixtures,

Supelco). For complex peaks, gas chromatography/mass spectrophotometry was used in a few random samples. All these methods showed that the methyl esters of eicosanoic acid (20:0) and gamma-linoleic acid (18:3 n-6) coincide, as do those of eicosaenoic acid (20:1 n-9) and alpha-linolenic acid (18:3 n-3). The relative amounts of the constituents in these complex peaks were not determined. The coefficient of variation in determining the percentages of the individual fatty acids changed with the level measured. It was between 0.9% and 4.4% for the major components (palmitic, palmitoleic, oleic and linoleic acids), whereas the error for trace constituents (less than 1%) was about 17.5%.

Statistics

Analysis of the results was performed by calculation of Spearman's linear correlation coefficients.

Results:

The results of the fatty acid analyses are expressed as a percentage of the total fatty acids. The majority of these are listed in Table 9:1 in ascending order of 17BHSD activity and similarly in Table 9:2 with the aromatase activities. Correlation coefficients were calculated between enzyme activities and the individual fatty acids, the ratio of polyunsaturated to saturated fatty acids (P/S ratio) and the total level of n-6 polyunsaturated fatty acids (total n-6, see below) in each sample.

Significant inverse correlations were noted between 17BHSD activity and the P/S ratio ($r = -0.73$, $p < 0.01$); linoleic acid ($r = -0.65$ $p < 0.05$); total n-6 ($r = -0.63$, $p < 0.05$); and eicosaenoic acid

TABLE 9:1.

FATTY ACID COMPOSITION AND 17βHSD ACTIVITY IN BREAST ADIPOSE TISSUE.

| Patient | 17βHSD pmol/mg prot/hr | Fatty Acid concentrations (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 20:0 | 20:1 | 20:2 | 20:3 | 20:4 | 22:4 | P/S ratio |
| 15 UIQ | 0.5 | 4.3 | 19.5 | 5.7 | 46.1 | 9.4 | .40 | 4.1 | .07 | .16 | .81 | .08 | .35 |
| 15 LOQ | 0.5 | 4.5 | 19.8 | 6.0 | 45.9 | 9.2 | .41 | 4.2 | .07 | .16 | .83 | .08 | .34 |
| 43 | 0.6 | 3.8 | 22.8 | 4.9 | 47.2 | 9.5 | .17 | 3.0 | .19 | .22 | .56 | .17 | .35 |
| 13 | 0.7 | 2.8 | 23.0 | 5.5 | 48.1 | 8.1 | .18 | 2.9 | .20 | .14 | .57 | .15 | .30 |
| 9 UOQ | 0.7 | 4.2 | 22.1 | 7.0 | 47.0 | 6.7 | .19 | 3.5 | .20 | .08 | .51 | .12 | .23 |
| 54 | 0.9 | 3.5 | 21.0 | 5.9 | 46.3 | 10.4 | .18 | 3.3 | .22 | .16 | .62 | .17 | .39 |
| 19 | 1.3 | 4.2 | 21.7 | 6.8 | 44.5 | 11.3 | .26 | 3.1 | .19 | .14 | .44 | .08 | .37 |
| 29(ben) | 1.5 | 4.2 | 20.6 | 8.6 | 45.6 | 10.3 | .28 | 3.0 | .13 | .14 | .52 | .08 | .33 |
| 37 | 3.5 | 2.8 | 21.5 | 3.1 | 50.6 | 7.5 | .08 | 2.9 | .19 | .20 | .48 | .25 | .32 |
| 9 UIQ | 3.6 | 3.9 | 22.6 | 5.6 | 48.3 | 7.0 | .13 | 3.0 | .08 | .11 | .44 | .13 | .25 |
| 6 | 4.2 | 4.6 | 22.5 | 6.5 | 46.7 | 7.4 | .17 | 2.7 | .13 | .14 | .49 | .11 | .25 |
| 33(ben) | 10.3 | 4.7 | 26.0 | 6.7 | 44.4 | 6.2 | .14 | 2.4 | .16 | .24 | .64 | .24 | .21 |

FATTY ACIDS - 14:0 myristic 18:1 oleic 20:1 gadoleic 20:4 arachidonic
16:0 palmitic 18:2 linoleic 20:2 eicosadienoic 22:4 docosatetraenoic
18:0 stearic 20:0 behenic 20:3 dihomo-V-linolenic

P/S ratio - ratio of total polyunsaturated to saturated fatty acids

(ben) - patient with benign breast disease

UOQ,UIQ,LOQ - adipose tissue from upper outer, upper inner, or lower inner breast quadrant.

TABLE 9:2.

FATTY ACID COMPOSITION AND AROMATASE ACTIVITY IN BREAST ADIPOSE TISSUE.

| Patient No. | Aromatase fmol/mg prot/hr | Fatty Acid concentrations (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 20:0 | 20:1 | 20:2 | 20:3 | 20:4 | 22:4 | P/S ratio |
| 33(ben) | 3.1 | 4.7 | 26.0 | 6.7 | 44.4 | 6.2 | .14 | 2.4 | .16 | .24 | .64 | .24 | .21 |
| 6 | 3.3 | 4.6 | 22.5 | 6.5 | 46.7 | 7.4 | .17 | 2.7 | .13 | .14 | .49 | .11 | .25 |
| 43 | 6.7 | 3.8 | 22.8 | 4.9 | 47.2 | 9.5 | .17 | 3.0 | .19 | .22 | .56 | .17 | .35 |
| 15 UIQ | 10.5 | 4.3 | 19.5 | 5.7 | 46.1 | 9.4 | .40 | 4.1 | .07 | .16 | .81 | .08 | .35 |
| 29(ben) | 11 | 4.2 | 20.6 | 8.6 | 45.6 | 10.3 | .28 | 3.0 | .13 | .14 | .52 | .08 | .33 |
| 9 UOQ | 12.2 | 4.2 | 22.1 | 7.0 | 47.0 | 6.7 | .19 | 3.5 | .20 | .08 | .51 | .12 | .23 |
| 37 | 14 | 2.8 | 21.5 | 3.1 | 50.6 | 7.5 | .08 | 2.9 | .19 | .20 | .48 | .25 | .32 |
| 13 | 16.4 | 2.8 | 23.0 | 5.5 | 48.1 | 8.1 | .18 | 2.9 | .20 | .14 | .57 | .15 | .30 |
| 19 | 34.2 | 4.2 | 21.7 | 6.8 | 44.5 | 11.3 | .26 | 3.1 | .19 | .14 | .44 | .08 | .37 |
| 9 UIQ | 34.9 | 3.9 | 22.6 | 5.6 | 48.3 | 7.0 | .13 | 3.0 | .08 | .11 | .44 | .13 | .25 |
| 15 LOQ | 35 | 4.5 | 19.8 | 6.0 | 45.9 | 9.2 | .41 | 4.2 | .07 | .16 | .83 | .08 | .34 |
| 54 | 40.9 | 3.5 | 21.0 | 5.9 | 46.3 | 10.4 | .18 | 3.3 | .22 | .16 | .62 | .17 | .39 |

FATTY ACIDS - 14:0 myristic 18:1 oleic 20:1 gadoleic 20:4 arachidonic
16:0 palmitic 18:2 linoleic 20:2 eicosadienoic 22:4 docosatetraenoic
18:0 stearic 20:0 behenic 20:3 dihomo-γ-linolenic

P/S ratio - ratio of total polyunsaturated to saturated fatty acids

(ben) - patient with benign breast disease

UOQ,UIQ,LOQ - adipose tissue from upper outer, upper inner, or lower inner breast quadrant.

($r = 0.61$, $p < 0.05$ ). These are illustrated in Figures 9:1-9:4. The same correlations are evident whether the enzyme activity is expressed as % conversion or as pmol/mg prot/hr. No other correlations reached statistical significance. There was no correlation between aromatase activity and any of the parameters. An example of the aromatase results is shown in Figure 9:5 where activity is plotted against linoleic acid concentration.

## Discussion

These results suggest a relationship between levels of polyunsaturated fatty acids in breast adipose tissue and 17BHSD activity. Since fatty acids in adipose tissue reflect the fatty acid composition of the diet[48,75,116], this implies that dietary fat could have a direct or indirect influence on 17BHSD. It is also possible, however that the two parameters are coincidentally related by another mechanism.

Dietary polyunsaturated fatty acids are required for the normal function of all tissues. Of the polyunsaturated fatty acids in the diet, linoleic acid is quantitatively most significant[56]. Although humans are able to synthesize many fatty acids from the saturated fats in the diet double bonds cannot be introduced into the n-3 and n-6 positions of the carbon chains. Humans are therefore unable to synthesize linoleic acid (18:2 n-6) or linolenic acid (18:3 n-3), which must be obtained from diet sources and are referred to as essential fatty acids. Further metabolism of these essential fatty acids produces two families (the n-3 and n-6) of essential fatty acids which are required for cell structures and prostaglandin synthesis[56,84].

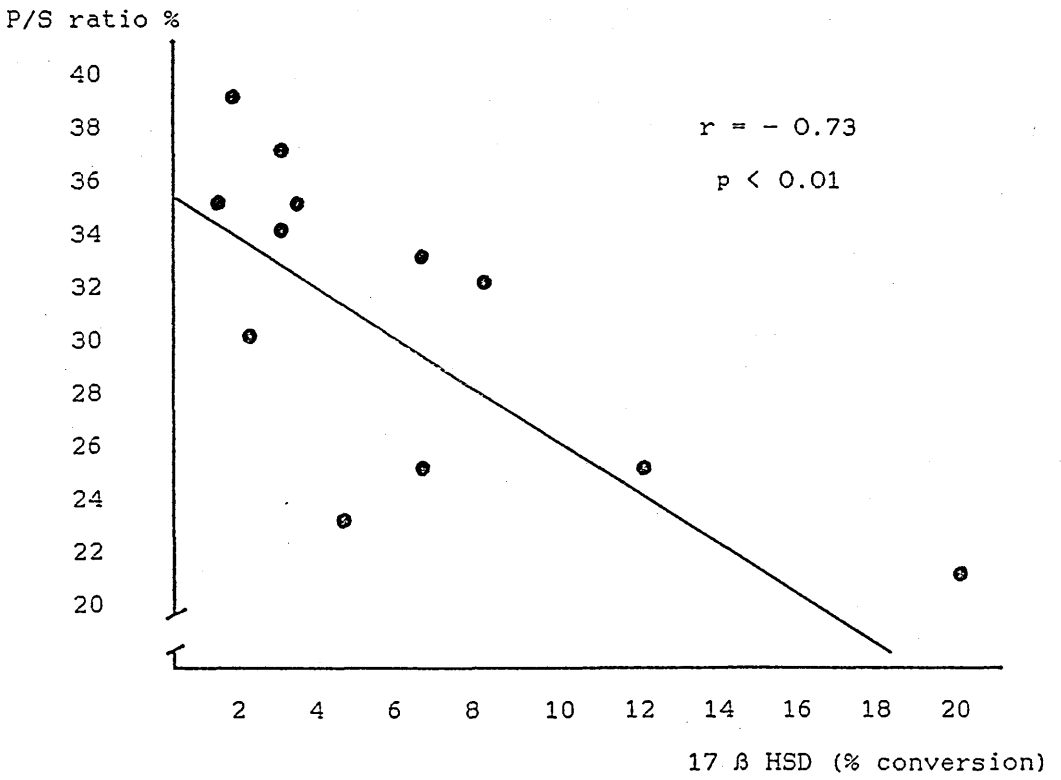



Figure 9:1.

Adipose tissue 17 ß HSD activity and the P/S ratio

(P/S ratio = ratio of total polyunsaturated to saturated fatty acids.)

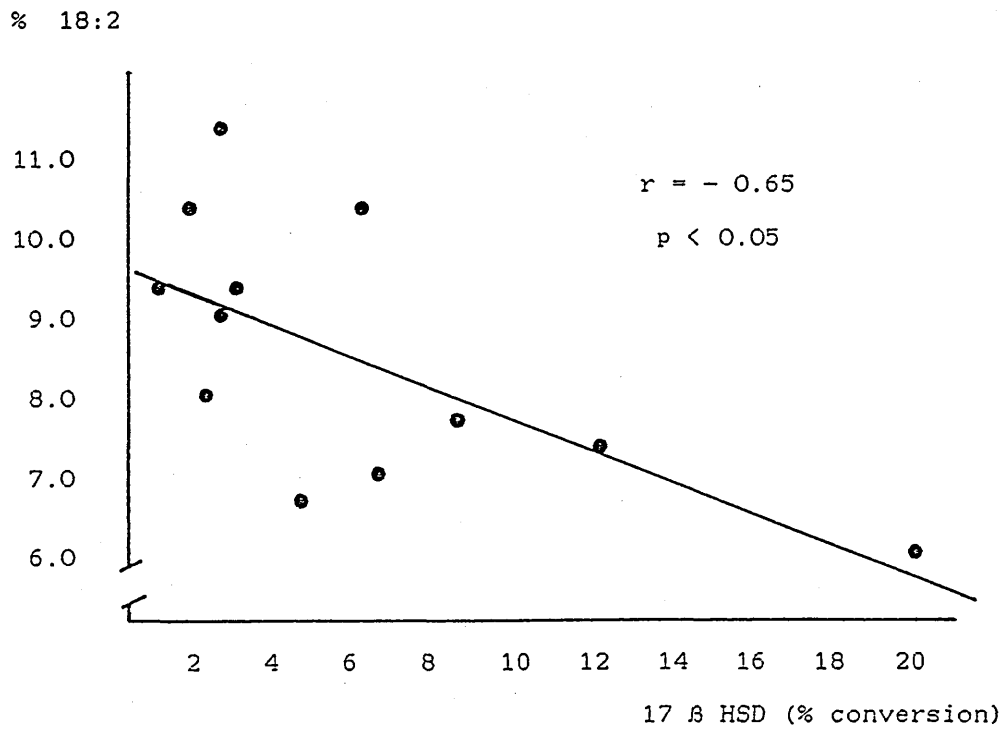



Figure 9:2.

Adipose tissue 17 ß HSD activity and linoleic acid concentration

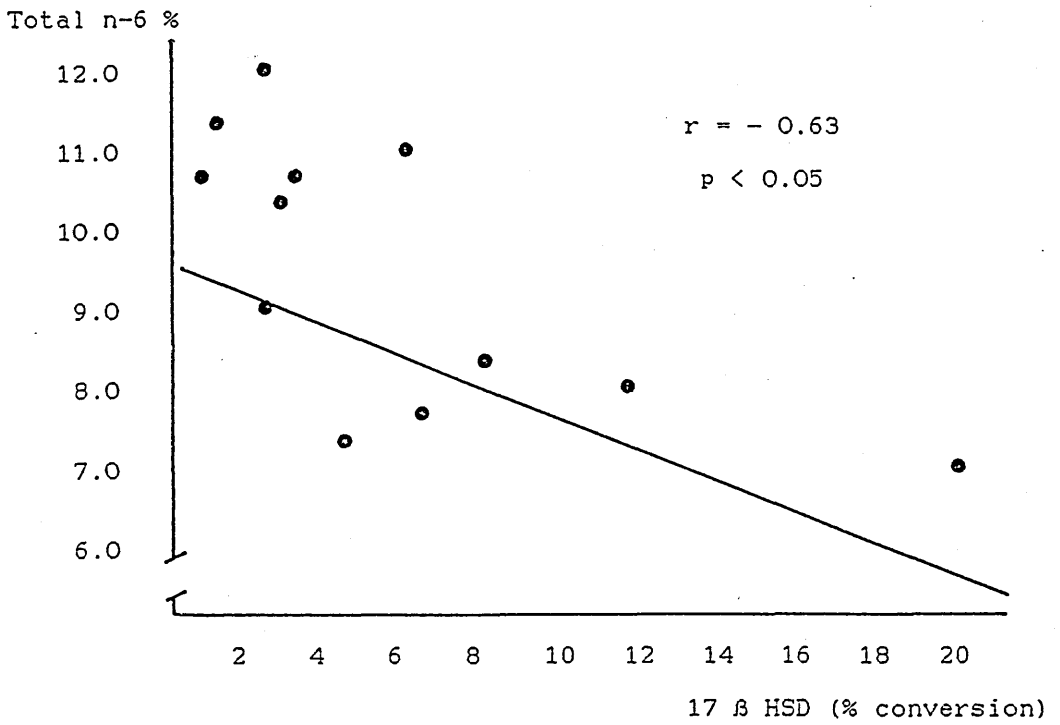



Figure 9:3.

Adipose tissue 17 ß HSD activity
and total n-6 PUFA concentration

Total n-6 = { 18:2 + 20:3 + 20:4 + 22:4 }

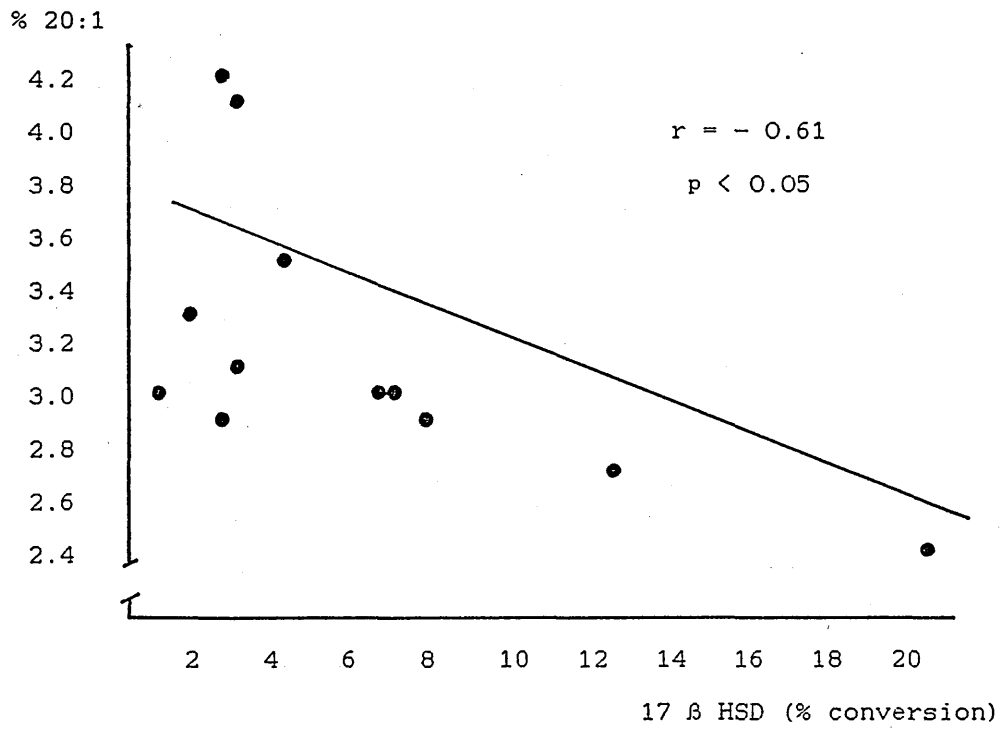



Figure 9:4.

Adipose tissue 17 ß HSD activity
and eicosaenoic acid concentration

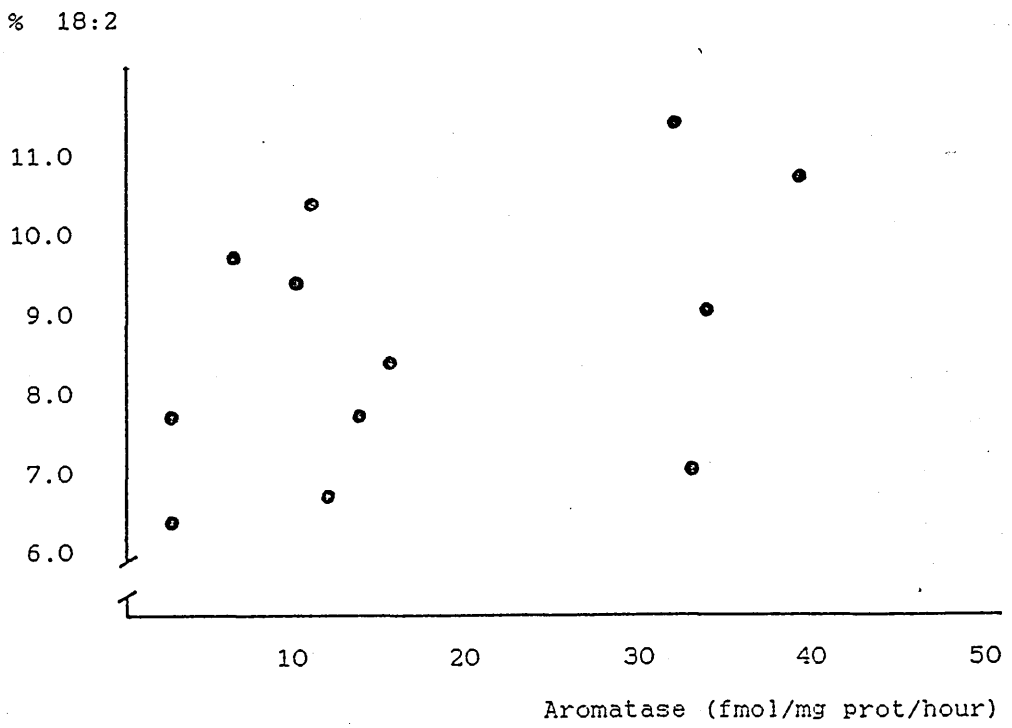



Figure 9:5.

Adipose tissue aromatase activity and linoleic acid concentration.

Since linoleic acid in the diet is present in much greater quantity than any of the other polyunsaturated fatty acids, and is also the precursor of the n-6 series of fatty acids, the total polyunsaturated fatty acids, the P/S ratio, and the total n-6 for each sample are largely a reflection of the level of linoleic acid. The correlations between these parameters and 17BHSD may therefore be simply a reflection of the correlation with linoleic acid. Linoleic acid however has no influence on the level of eicosaenoic acid (20:1 n-9)[56], levels of which also correlate inversely with 17BHSD.

There are a number of possible explanations for the observed relationships. Levels of fat in the diet can significantly effect cellular metabolism by altering levels of cytochromes in the endoplasmic reticulum[117]. Such changes could influence 17BHSD activity by altering the synthesis of the enzyme and/or essential cofactors.

Alternatively polyunsaturated fatty acids could have an indirect effect on enzyme activity by modifying the pathways which control enzyme expression. As mentioned above, 17BHSD activity in adipose tissue and in breast epithelial cells appears to be influenced by androgens, progesterone, Epidermal Growth Factor and Transforming Growth Factor alpha. Free fatty acids are known to influence the availability of steroid hormones for biological activity[23]. Levels of free fatty acids in adipose tissue might therefore modify the effect of androgens and progesterone on 17BHSD activity. Epidermal Growth Factor and Transforming Growth Factor alpha act via cell

membrane receptors[93]. Since polyunsaturated fatty acids are essential components of cell membrane phospholipids[56], subtle changes in the fatty acid composition of these phospholipids could have considerable influence on the regulation of membrane receptors[26] and subsequent signal transduction mechanisms[85,165] thereby modifying the effect of growth factors on 17BHSD activity.

The earlier studies indicated that enhanced levels of 17BHSD were found around large tumours. Perhaps the observed association is not specifically between fatty acids and 17BHSD, but between fatty acids and large tumours. This explanation seems unlikely since two of the samples came from women with benign breast disease and a further two samples were from breast quadrants which did not contain tumours. For the other eight samples there is no significant relationship between tumour size and the fatty acid levels. Nevertheless tumours require essential fatty acids for cell membrane synthesis and for the maintenance of normal membrane function[166]. It has been suggested that a significant proportion of the lipid incorporated into tumours is derived from surrounding adipose tissue[116]. Large tumours are therefore likely to absorb essential fatty acids from their surrounding tissues, and this could result in lower levels of essential fatty acids in the adipose tissue around these tumours. Such a mechanism could contribute to the association between high 17BHSD activity and low levels of essential fatty acids.

Animal experiments suggest that polyunsaturated fatty acids stimulate tumour growth by providing the precursors for prostaglandins[25], which have been implicated as important local

growth factors in mammary tumour cells[8,9]. At present there is no evidence that prostaglandins directly influence 17BHSD but they could play a role in regulation of the enzyme.

The absence of any correlation between fatty acid levels and aromatase activity suggests that dietary fat does not influence this enzyme system. This study, however, has only considered the relative proportions of the various fatty acids in adipose tissue. It is possible that the composition of membrane phospholipids or the total amount of fat in the diet could be important factors. High fat diets are known to increase tissue levels of Cytochrome $p450^{117}$ which is a prosthetic group for the aromatase complex and could be crucial in determining activity in vivo. Further studies are required before any firm conclusion can be reached on the influence of dietary fat on aromatase activity.

Nevertheless, the interesting correlations between fatty acids and 17BHSD activity suggest that dietary fat could influence some aspects of steroid metabolism in breast adipose tissue.

# Chapter 10

Discussion of the relevance of steroid metabolism in breast adipose tissue.

The role which steroid hormones play in the natural history of breast cancer is a problem which has attracted the attention of research investigators for many years. Oestrogens have come under particular scrutiny[77], since there is indirect evidence that altered oestrogen exposure may influence the incidence of the disease; e.g. the diminished risk conferred by early castration[45,92] and the increase in risk associated with prolonged oestrogen administration[19,20,81].

The precise role played by oestrogens in the evolution of breast cancer is still the subject of considerable debate[77] but current thinking is that excessive or prolonged oestrogenic stimulation of target cells in the breast may render them more susceptible to carcinogens such as irradiation or chemicals in the diet which then cause malignant transformation[90,133,155]. Oestrogens would thus be acting more as a promoter, "setting the scene for carcinogenesis" than as an inducer or carcinogen. In established breast cancers, studies on endocrine therapy have clearly indicated the importance of oestrogens in the mairtenance of tumour growth[71]. In terms of circulating hormones however, there has been little unequivocal evidence for any consistent abnormality in plasma levels of oestrogen or oestrogen excretion in women with breast cancer[87].

These findings, however, do not rule out the possibility that the breasts of these women have been exposed to abnormal quantities of oestrogen. While circulating hormones may be the major source of oestrogens or their precursors, local factors within the breast may be more important in determining endogenous concentrations of oestrogen within the breast and the degree to which breast

epithelial cells are exposed to oestrogenic stimuli.

In support of this, the concentrations of most steroid hormones in breast tissues have been shown to be higher than those in plasma[37,46]. There appears to be no consistent relationship, however, between plasma and tissue levels. This inconsistency suggests that variable local factors such as the selective uptake of steroids from the circulation or local synthesis and metabolism determine tissue concentrations[47]. Such local events are likely to be of particular relevance in postmenopausal women, in whom the aromatisation of androgens within peripheral tissues is the principal source of oestrogen[69].

The factors which govern the differential uptake of stercids into peripheral tissues are largely unknown. With regard to oestrogen synthesis however, aromatase and 17BHSD activity are critical control points[87]. Evidence for involvement of these enzymes in determining local concentrations of hormones comes from the infusion studies of Reed et al[132]. These show that intravenous infusion of radiolabeled androstenedione is followed by the appearance of labeled oestrone in breast tissues. After infusion of labeled oestrone, labeled oestradiol is detectable in breast tissues[99,132].

Within peripheral tissues therefore, there appears to be a potential microendocrine system to which hormone sensitive cells may be exposed and may respond. The possible contribution of breast adipose tissue to such metabolism is affirmed in the present studies by the demonstration of both aromatase and 17BHSD activity

in all samples of breast adipose tissue examined. In these studies and those of others[10,37,121], enzyme activities varied widely between individuals and between different body sites. These enzymes might therefore contribute to the wide variation in oestrogen levels in peripheral tissues and between different parts of the breast.

The present studies have shown that aromatase and 17BHSD activity both vary widely between the quadrants of cancerous breasts. Since it is rarely possible to obtain fresh samples of tissue from multiple quadrants of normal or non-cancerous breasts it will be difficult to determine whether steroid metabolism also varies throughout normal breasts. It is therefore conceivable that significant variation in enzyme activity only occurs in those breasts which either contain malignant tumours or are likely to develop such tumours.

The present studies have shown that enhanced aromatase activity in breast adipose tissue is associated with the presence of breast cancer, but not with any specific features of the tumours. In contrast enhanced 17BHSD activity is not specifically associated with breast cancer but is correlated with features of the tumours such as size, nodal metastases and response to treatment.

If local aromatase activity determines local tissue concentrations of oestrogen then the association of enhanced aromatase activity in breast adipose tissue with breast cancer is consistent with the hypothesis that increased exposure to oestrogens may be an

important factor in the early evolution of breast cancer. The lack of correlation between aromatase activity in adipose tissue and the tumour type, stage or oestrogen receptor activity suggests that local aromatase activity may be a less significant factor in the continued growth and dissemination of most of the tumours, once established.

In contrast, levels of 17BHSD activity in breast adipose tissue do not differ between cancer patients and patients with benign breast disease and levels of activity in breast quadrants do not correlate with tumour location implying that activity of this enzyme may not be crucial in the early stages of tumour development. The correlation between levels of 17BHSD and tumour size, nodal metastases and response to treatment suggest that this enzyme may be more important in the continued growth and dissemination of established tumours.

It remains to be determined whether tumours have a significant effect on enzyme activities in their surrounding tissues. Tumours could influence aromatase and 17BHSD activity selectively by producing factors which increase or inhibit levels of the enzymes or essential cofactors. Alternatively enzyme activity could be affected indirectly as a consequence of other effects of tumours on their surrounding tissues. Tumours are known to produce growth factors which may modulate enzyme activity in peripheral tissues[100,101]. These include factors which may be produced in a paracrine manner by tumours[93] and preliminary results indicate that the addition of extracts from breast cancers to cultures of adipose tissue may stimulate oestrogen biosynthesis[130] Some tumours

however also produce factors which increase the vascularity of their immediate surroundings (angiogenesis factors)[18]. An increase in vascularity could also lead to an increase in the numbers of stromal cells which are thought to be the principal site of aromatase activity[29]. Tumours may also extract nutrients and cofactors from their local environment[116]. Such substances may be important determinants of local steroid metabolism either as essential cofactors for the metabolic pathways or as regulators/inhibitors of activity.

The present studies can neither confirm nor refute the possibility that tumours influence local metabolism. What can be inferred from the results in Chapter 7 however, is that there is no consistent trend for either enhancement or inhibition.

It may be possible to determine whether tumours can influence metabolism in their surrounding tissues by measuring enzyme activities in tissue samples obtained before and after local excision or systemic treatment of tumours. Such studies should indicate whether tumour excision or regression results in significant alteration of local metabolism. Confirmation that any changes which occur are due to local effects of the tumour could come from parallel in vitro studies where tumour extracts are added to cell cultures of adipose tissue.

Even if tumours do influence metabolism in their immediate vicinity, the wide variation in activity between different body sites and different breast quadrants in some individuals indicates that other factors must be involved in enzyme control.

In view of this local variation in enzyme activities, it is not surprising that there is no apparent relationship between enzyme activity and age, menopausal status, height, weight, obesity , parity, age at menarche, age at first full term pregnancy, or family history of breast cancer. Such local variation in any one individual suggests that there must be local tissue factors which influence enzyme activity.

Variable cellularity is unlikely to account for the observed variations since the assay results were all corrected for the protein concentration of the tissue extracts. This should compensate for variations in cellularity.

Although a number of factors have been identified which will modify in vitro steroid metabolism (corticosteroids[103], prolactin[54], androgens[16,37], cyclic AMP[148], transforming growth factor alpha[100], epidermal growth factor[100] and methyl xanthines[148]) there is little evidence that any of these factors operate in vivo[87]. Unfortunately most of these studies have attempted to correlate tissue levels of enzyme activity with circulating levels of these factors. There may be little correlation between plasma and tissue levels of such substances. Additionally, if these agents do influence local enzyme activity then there must be tissue differences in levels of the factors to account for the variation in activity seen between different body sites in the same individual. Further investigation into the control of local enzyme activities should concentrate on tissue concentrations of factors rather than circulating levels.

The results in Chapter 9 suggest a relationship between levels of polyunsaturated fatty acids in breast adipose tissue and 17BHSD activity. Since fatty acids in adipose tissue reflect the fatty acid composition of the diet[48,75,116] this implies that dietary fat could have a direct or indirect influence on 17BHSD. If tissue samples can be obtained from different populations with substantial differences in dietary fat intake it should be possible to determine if diet is an important factor.

Although the results from these studies do not provide conclusive proof for the involvement of local metabolism in the evolution and progression of breast cancer, the results are consistent with potentially significant interactions between the tumour and their surrounding tissues. If such interactions do occur, they may be of relevance to the management of breast cancer.

It is well recognised that, in the majority of patients, breast cancer is a systemic disease by the time it is clinically detectable[59]. Surgical treatment will therefore only cure a small proportion of patients. To have any impact on the morbidity and mortality from this disease, an effective means of preventing the disease must be devised or effective systemic therapy must be developed.

The suggestion that local aromatase activity may be important in the early evolution of breast cancer assumes that the observed levels of aromatase activity have been present for a number of months or years. Whether enzyme activity in breast adipose tissue

remains constant for prolonged time periods has yet to be established. It may be possible to measure enzyme activity in serial samples of tissue obtained from patients who undergo successive breast biopsies. However, given the variation that can occur in enzyme activity within one breast quadrant it is likely to be extremely difficult to prove whether activity is consistent with time.

If enhanced aromatase activity in breast adipose tissue is important in the evolution of breast cancer, then the patients with benign breast disease who had relatively high activity should be at increased risk of developing cancer in the future. These patients should therefore be followed closely to determine whether they do subsequently develop tumours.

The results in Chapters 3 & 5 suggest that locally enhanced aromatase activity may be important in the evolution of breast cancers. If the factors which control local aromatase can be elucidated and are amenable to modification, reduction of activity could reduce the incidence of breast cancer or at least delay its appearance.

The effectiveness of aromatase inhibitors such as aminoglutethimide in producing tumour regression[21] confirms the importance of oestrogen biosynthesis in the continued growth of some breast cancers. Whether local activity in breast adipose tissue makes a significant contribution to the growth of tumours still remains to be confirmed. Nevertheless the responses to aminoglutethimide seen in two patients with higher than average aromatase activity

suggests that such activity could be important or at least provides an indicator of the capacity for peripheral biosynthesis in these patients. This explanation is supported by the low aromatase activity observed in breast adipose tissue from the patient who failed to respond to aminoglutethimide.

It is notable that both patients who responded to aminoglutethimide had high levels of oestrogen receptor protein in their tumours whereas the patient who failed to respond had a low oestrogen receptor protein level. It is well established that oestrogen receptor positive tumours are more likely to respond to hormonal manipulation[73]. However, not all oestrogen receptor positive tumours respond to aminoglutethimide[21], and it is possible that measurement of both oestrogen receptor protein level and aromatase activity would enable better selection of patients for such therapy.

Response to the anti-oestrogen tamoxifen correlates well with the oestrogen receptor status of breast tumours[73], although again not all oestrogen receptor positive tumours respond. The observation of higher levels of aromatase and 17BHSD activity in patients who failed to respond to tamoxifen suggests that the local microendocrine environment around tumours may be able to overcome the effects of tamoxifen. Perhaps such patients would be more likely to respond to an aromatase inhibitor rather than an anti-oestrogen.

Enhanced 17BHSD activity appears to be associated with tumours which have a poor prognosis. The interesting correlation between

local levels of 17BHSD and response to therapy suggests that measurement of such activity could provide further helpful information for determining which patients are likely to respond to systemic therapy.

Why reduced activity of this enzyme should predict responsiveness of a tumour to chemotherapy is not readily explanable. Perhaps locally elevated activity influences tumour metabolism to confer resistance to therapy. Further study of the relationship between activity of this enzyme and tumour behaviour is needed to clarify the position, but if enhanced activity does promote tumour growth and resistance to therapy then inhibition or reduction of activity might render these tumours susceptible to treatment.

The results from these studies have therefore raised a number of questions that now require to be answered in order to clarify the role of local steroid metabolism in the natural history of breast cancer. The correlations seen in these studies suggest that such metabolism may be important in the development and continued growth of breast cancers. With greater understanding of the significance of local metabolism and the factors which influence activity it may be possible to develop more logical and effective systemic therapy and even influence the natural history of the disease.

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TABLE A:1 DETAILS OF BREAST CANCER PATIENTS STUDIED.

| No. | AGE | MENOP. STATUS | PARITY | FAMILY HISTORY | HEIGHT (CM) | WEIGHT (Kg) | QUETLET INDEX |
|---|---|---|---|---|---|---|---|
| 1 | 50 | Peri | 2+0 | - | - | - | - |
| 2 | 61 | Post | 2+0 | - | - | - | - |
| 3 | 65 | Post | 1+0 | - | 155 | 62.6 | 26.1 |
| 4 | 63 | Post | 2+1 | + | 153 | 69.9 | 29.9 |
| 5 | 54 | Post | 2+2 | - | 163 | 78.9 | 29.7 |
| 6 | 50 | Peri | 3+0 | - | 163 | 76.4 | 28.7 |
| 7 | 31 | Pre | - | - | - | - | - |
| 8 | 45 | Pre | 0+0 | - | 165 | 66.7 | 24.5 |
| 9 | 76 | Post | 3+0 | - | 163 | 74.4 | 28.1 |
| 10 | 48 | Pre | 1+0 | - | 160 | 89 | 34.8 |
| 11 | 31 | Pre | 1+0 | + | 155 | 57.2 | 23.8 |
| 12 | 39 | Pre | 2+0 | + | - | - | - |
| 13 | 36 | Pre | 4+0 | - | 158 | 71.2 | 28.5 |
| 14 | 61 | Post | 2+0 | - | 155 | 66.7 | 27.8 |
| 15 | 66 | Post | 8+0 | - | 173 | 63.5 | 21.2 |
| 16 | 45 | Pre | 2+2 | + | 165 | 57.2 | 21.0 |
| 17 | 64 | Post | 2+0 | - | 163 | 58.1 | 22.0 |
| 18 | 61 | Post | 5+0 | - | 163 | 68.2 | 25.8 |
| 19 | 34 | Pre | 2+0 | - | 168 | 68.0 | 24.2 |
| 20 | 68 | Post | 2+1 | - | 160 | 54.0 | 21.1 |
| 21 | 58 | Post | 1+1 | - | 163 | 58.1 | 22.0 |
| 22 | 46 | Pre | 3+0 | + | - | - | - |
| 23 | 75 | Post | 2+0 | - | 150 | 50.8 | 22.6 |
| 24 | 49 | Peri | 3+1 | - | 149 | 54.0 | 24.5 |
| 25 | 53 | Post | - | - | - | 63.5 | - |
| 26 | 34 | Pre | 2+0 | + | 179 | 73.5 | 22.9 |
| 27 | 50 | Pre | 2+1 | - | 165 | 70.0 | 25.7 |
| 28 | 43 | Pre | 3+0 | - | 163 | 50.8 | 19.2 |
| 29 | 53 | Pre | 3+0 | + | - | - | - |
| 30 | 59 | Post | 1+1 | - | - | - | - |
| 31 | 61 | Post | 1+0 | - | 158 | 65.3 | 26.3 |
| 32 | 55 | Peri | 2+0 | - | - | - | |
| 33 | 50 | Peri | 0+1 | - | 174 | 57.6 | 19.0 |
| 34 | 62 | Post | 3+0 | + | 155 | 59.9 | 24.9 |
| 35 | 45 | Pre | 0+0 | - | 165 | 53.5 | 19.7 |
| 36 | 51 | Peri | 0+0 | - | 151 | 47.3 | 20.8 |
| 37 | 40 | Pre | 2+0 | - | 158 | 74.0 | 29.8 |
| 38 | 76 | Post | - | - | - | - | - |
| 39 | 66 | Post | 0+0 | - | 160 | 82.5 | 32.2 |
| 40 | 63 | Post | 2+0 | + | 166 | 71.5 | 25.9 |
| 41 | 86 | Post | - | - | - | - | - |
| 42 | 53 | Peri | 3+1 | - | 153 | 63.5 | 27.3 |
| 43 | 73 | Post | 2+0 | + | - | - | - |
| 44 | 58 | Peri | 0+0 | + | 165 | 63.5 | 23.3 |
| 45 | 39 | Pre | 0+0 | - | 158 | 66.7 | 26.7 |
| 46 | 50 | Pre | 4+0 | - | - | - | - |
| 47 | 50 | Pre | 2+0 | - | 160 | 70.0 | 27.3 |
| 48 | 50 | Pre | 0+0 | - | 164 | 91.4 | 34.0 |
| 49 | 34 | Pre | 2+0 | + | - | - | - |
| 50 | 33 | Pre | 1+2 | + | 158 | 57.2 | 23.1 |
| 51 | 54 | Post | 0+0 | - | 154 | 66.0 | 27.8 |

Table A:2

DETAILS OF PATIENTS WITH BENIGN BREAST DISEASE.

| Number | AGE | MENOP. STATUS | PARITY | FAMILY HISTORY | DRUG HIST | HEIGHT (CM) | WEIGHT (Kg) | QUETLET INDEX |
|---|---|---|---|---|---|---|---|---|
| 1 | 45 | Pre | 1+1 | + | - | 168 | 64.4 | 22.8 |
| 2 | 65 | Post | 3+1 | + | - | 163 | 70.0 | 26.5 |
| 3 | 44 | Pre | 2+1 | - | - | 166 | 61.7 | 22.4 |
| 4 | 38 | Pre | 2+0 | - | - | 158 | 59.0 | 23.8 |
| 5 | 31 | Pre | 0+0 | - | - | 160 | 70.8 | 27.7 |
| 6 | 32 | Pre | 1+1 | - | - | 155 | 57.2 | 23.8 |
| 7 | 41 | Pre | 1+0 | - | - | - | - | - |
| 8 | 50 | Pre | 0+0 | + | - | 156 | 63.5 | 26.1 |
| 9 | 43 | Pre | 3+0 | - | - | - | - | - |
| 10 | 49 | Pre | 2+0 | - | - | 158 | 58.5 | 23.6 |
| 11 | 33 | Pre | 0+1 | - | - | 163 | 56.2 | 21.3 |
| 12 | 65 | Post | 1+1 | - | - | 164 | 53.5 | 19.9 |
| 13 | 34 | Pre | - | - | - | - | - | - |
| 14 | 49 | Pre | 3+0 | - | - | 168 | 60.8 | 21.6 |
| 15 | 41 | Pre | 3+0 | - | - | 152 | 55.3 | 23.8 |
| 16 | 29 | Pre | 1+0 | + | - | 160 | 57.2 | 22.3 |
| 17 | 54 | Post | 1+1 | - | + | 159 | 63.5 | 25.3 |
| 18 | 37 | Pre | 3+1 | - | - | 168 | 59.4 | 21.2 |
| 19 | 59 | Post | 4+0 | - | - | - | - | - |
| 20 | 45 | Pre | 0+0 | + | - | 171 | 61.7 | 21.1 |
| 21 | 49 | Pre | 3+0 | - | - | 167 | 70.0 | 25.1 |
| 22 | 36 | Pre | 2+0 | - | - | 173 | 70.0 | 23.4 |
| 23 | 54 | Post | 3+0 | - | - | 155 | 57.2 | 23.8 |
| 24 | 54 | Post | 2+0 | - | - | - | - | - |
| 25 | 46 | Pre | 1+0 | - | - | 161 | 52.2 | 20.1 |
| 26 | 51 | Pre | 4+0 | + | - | 171 | 70.0 | 23.9 |
| 27 | 39 | Pre | 2+1 | - | - | - | - | - |
| 28 | 53 | Peri | 2+0 | - | - | - | - | - |
| 29 | 49 | Peri | 1+0 | - | - | 164 | 67.5 | 25.1 |
| 30 | 33 | Pre | 2+1 | - | - | 171 | 70.0 | 23.9 |
| 31 | 43 | Pre | 2+0 | - | - | 173 | 57.6 | 19.3 |
| 32 | 31 | Pre | 3+1 | + | - | - | - | - |
| 33 | 63 | Post | 2+0 | - | - | - | - | - |
| 34 | 47 | Pre | - | - | - | - | - | - |
| 35 | 27 | Pre | 0+0 | - | - | - | - | - |

Table A:3 CLINICAL DETAILS OF BREAST CANCER PATIENTS STUDIED

(Tumour size = pathologist's measurement, figure in brackets = clinical measurement before systemic therapy)

| No. | Tumour stage | Size (cm) | Location | Histology | ER level (fmol/mg) | Treatment | Node Status |
|---|---|---|---|---|---|---|---|
| 1 | T2No | 3.4 + 2 | U+LOQ's | Inv Ca + In situ Ca | 3 + 2 | Mx + Cl | +ve 10/17 |
| 2 | T2N1b | 2.0 (4.5) | UOQ | Inv Ca + In situ Ca | 62 | Tam. > resp > Mx + Cl | +ve 1/8 |
| 3 | T2No | 3.2 | LOQ | Inv Ductal Ca | 122 | Mx + NS + R/TH | +ve 1/6 |
| 4 | T2No | 4.5 | 6 o'c | Inv Ductal Ca | 330 | Mx + Cl | +ve 4/13 |
| 5 | T2No | 3.0 | UOQ | Inv Lobular Ca | 0 | Mx + Cl | +ve 1/13 |
| 6 | T2N1b | 3.6 | 12 o'c | Inv Mucoid Ca | 32 | WLE + ANS | +ve 3/5 |
| 7 | T1N1b | 1.3 | - | Scirrhous Ca | 21 | WLE + ANS | +ve 1/4 |
| 8 | Bilat | 1.8 + 0.9 | LUO RUI | L Inv Ca, R Cribriform | L 193 R 70 | Bilat WLE | -ve |
| 9 | T4N1a | 6.0 | UIQ | Inv Lobular Ca | 25 | Tam. no resp > Mx + Cl | -ve |
| 10 | T3No | 2.0 (5.8) | L 3 o'c | Inv Ductal Ca | 20 | oox. no resp, CHOP > resp >Mx | -ve |
| 11 | T1No | 1.0 | UOQ | Inv Ductal Ca | - | Mx + Cl | -ve |
| 12 | T1N2 | 1.5 | LOQ | Inv Mucoid Ca | 23 | WLE + R/TH > Mx | +ve |
| 13 | ToNo | 1.5 | L 9 o'c | Inv Tubular Ca | 92 | Mx + Cl | -ve |
| 14 | T3No | 2.6 (5.2) | UOQ | Adenocarcinoma | 167 | AMG > resp > Mx + Cl | -ve |
| 15 | TisNo | 0.8 | L 3 o'c | Intraduct Ca | 245 | Mx | -ve |
| 16 | T3No | 5.0 | UOQ | Inv Mucoid Ca | 93 | Oox > resp > Mx + Cl | -ve |
| 17 | T4No | 2.5 (4.0) | Central | Inv Ductal Ca | 129 | Tam > resp > Mx + Cl | -ve |
| 18 | T3No | 5.2 (4.0) | 12 o'c | Inv Lobular Ca | 0 | CHOP (defaulted) > Mx + Cl | -ve |
| 19 | T2No | 2.8 | 12 o'c | Inv Ductal Ca | 2 | WLE + ANS | -ve |
| 20 | ToN1a | - | - | Paget's + In situ Ca | 0 | Mx + ANS | -ve |
| 21 | T4N1b | 1.9 | Central | Inv Ductal Ca | 6 | Mx + Cl | -ve |
| 22 | T4No | 5.0 | UIQ | Inv Ca | 18 | CHOP + R/TH > Mx + Cl | -ve |
| 23 | T2No | 4.5 (3.2) | L 3 o'c | Inv Ductal Ca | 0 | Tam > progression > Mx + Cl | +ve 7/24 |
| 24 | T1No | 1.2 | UOQ | Inv Ca | 118 | Mx + Cl | -ve |
| 25 | T2No | 3.5 | 12 o'c | Inv Ca | 486 | Mx | ? |
| 26 | T3N1b | 0 (10.0) | UOQ | Adenoca. | 2 | CHOP > Comp. Resp > Mx | -ve |
| 27 | ToNo | 0.6 | UOQ | Inv Ca | 23 | WLE + ANS | -ve |
| 28 | T4N1b | 3.0 | LIQ | Inv Ca | 0 | CHOP > Comp. Resp > Mx | -ve |
| 29 | ToMo | 1.0 | UOQ | Inv Ca | 0 | Mx + Cl | -ve |
| 30 | T2No | 0 (4.2) | UOQ | Inv Ductal Ca | 6 | AMG >Prog. Dis.>CHOP >CR > Mx | -ve |
| 31 | T2N1b | 2.0 (4.5) | 6 o'c | Inv Ductal Ca | 0 | Tam >Prog. Dis.>CHOP >PR > Mx | +ve 7/13 |
| 32 | ToNo | 0 | - | In situ Ca | - | Mx + ANS | -ve |
| 33 | Trecur | 5.0 | 3-9s | Inv ca + In situ | 0 | R/TH for in situ Mx for recur. | -ve |
| 34 | T2N1b | 3.2 | UOQ | Inv Ductal Ca | 789 | Mx + Cl | +ve 10/23 |
| 35 | T2N1b | 2.8 | 6 o'c | Inv Tubular Ca | 51 | Mx + Cl | -ve |
| 36 | T3N1b | 1.5 (6.2) | UIQ | Inv Ca | 19 | CHOP > resp > Mx + Cl | +ve 2/13 |
| 37 | T2No | 2.5 | UOQ | Inv Ductal Ca | 0 | Mx + Cl | +ve 3/4 |
| 38 | - | 1.2 | - | Anaplastic Ca | - | WLE | - |
| 39 | T2No | 2.5 | UOQ | Inv Lobular variant | 27 | Mx + Cl | -ve |
| 40 | T3N1b | 5.0 | UOQ | Inv Ductal Ca | 7 | Mx + Cl (AMG stopped -reaction) | +ve 20/29 |
| 41 | T2N1a | 3.6 | UOQ | Inv Mucoid Ca | 1072 | WLE | - |
| 42 | T3No | 2.0 (5.2) | UOQ | Inv Ductal Ca | 227 | AMG > resp > Mx + Cl | -ve |
| 43 | T2N1b | 1.8 (4.3) | 12 o'c | Inv Ductal Ca | 1151 | Tam > resp > Mx + Cl | +ve 3/14 |
| 44 | T4N3 | 7.0 | Central | Adenocarcinoma | 19 | Biopsy > CHOP + R/TH | +ve N3 |
| 45 | T3No | 3.0 (5.2) | UIQ | Atypical Medullary Ca | 0 | Oox >no resp >Chop >resp >Mx | +ve 4/11 |
| 46 | T2No | 2.0 | LOQ | Inv Ca | 71 | Mx + Cl | +ve 10/17 |
| 47 | T4N1a | 3.7 | UOQ | Spindle Cell Ca | 9 | CHOP > no resp > Mx +Cl | -ve |
| 48 | T2No | 2.8 | LIQ | Inv Ca | 21 | Mx + Cl | -ve |
| 49 | T1No | 0.7 | UOQ | Inv Ca | 62 | Mx + Cl | +ve 2/14 |
| 50 | T2N1a | 2.9 | 12 o'c | Inv Ductal Ca | 0 | WLE | - |
| 51 | T2No | 3.8 | UIQ | Inv Ductal Ca | 0 | Mx + ANS | -ve 1 node |

Table A:4

Histological details of benign breast biosies.

| Number | Location | Histology |
|---|---|---|
| 1 | LIQ | Fibroadenoma |
| 2 | LIQ | Fat and fibrous tissue |
| 3 | R 3 o'c | Benign mammary dysplasia |
| 4 | R 3 o'c | Fibrocystic disease, fibroadenomatoid nodule |
| 5 | UOQ | Fibroadenoma |
| 6 | 6 o'c | Benign mammary dysplasia, fibroadenomatoid nodule |
| 7 | LOQ | Within normal limits (breast reduction) |
| 8 | UOQ | Benign mammary dysplasia with hyperplasia |
| 9 | OUQ | Mammary dysplasia, adenosis, sclerosis |
| 10 | OUQ | Apocrine cysts |
| 11 | UOQ | Fibrocystic disease |
| 12 | UOQ | Apocrine cyst, mammary dysplasia |
| 13 | LOQ | Pregnancy changes |
| 14 | 12 o'c | Involutional fibrosis |
| 15 | LOQ | Mammary dysplasia |
| 16 | UOQ | Within normal limits |
| 17 | UIQ | Cyst, mammary dysplasia |
| 18 | UOQ | Dysplasia, peri-ductal mastitis, cysts |
| 19 | UOQ | Mammary dysplasia |
| 20 | Central | Cysts, sclerosis |
| 21 | UOQ | Mammary dysplasia, hyperplasia |
| 22 | 12 o'c | Dysplasia, fibrosis, cysts, hyperplasia |
| 23 | UOQ | Fibrocystic disease |
| 24 | UOQ | Fibrocystic disease |
| 25 | UIQ | Fibroadenoma |
| 26 | UIQ | Dysplasia, apocrine cysts |
| 27 | UIQ | Within normal limits |
| 28 | L 3 o'c | Cysts |
| 29 | UOQ | Sclerosing adenosis |
| 30 | UOQ | Fibrosis, hyperplasia |
| 31 | R 9 o'c | Fibrocystic disease |
| 32 | UOQ | Dysplasia, hyperplasia |
| 33 | 12 o'c | Fat necrosis |
| 34 | LIQ,LOQ | Normal (breast reduction) |
| 35 | LIQ,LOQ | Normal (bilateral reduction) |

Table B:1

AROMATASE ACTIVITY IN ADIPOSE TISSUE FROM BREAST CANCER PATIENTS.

| No. | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | AROMATASE %CONV. | AROMATASE fmol/mg prot /hour |
|---|---|---|---|---|
| 1 | 0.8 | 0.9 | .033 | 24.4 |
| 2 | 1.1 | 1.8 | .073 | 27 |
| 3 | 1.1 | 1.2 | .132 | 70.4 |
| 4 | 2 | 1.6 | .036 | 15 |
| 5 | 2 | 1.2 | .09 | 50 |
| 6 | 2 | 2.9 | .128 | 29.4 |
| 7 | 1.2 | 4.7 | .362 | 51.4 |
| 8Bil | 0.9/1.2 | 3.5/2.2 | .222/.051 | 42.3/15.5 |
| 9 | 2.1 | 1.3 | .067 | 34.9 |
| 10 | 2 | 1.1 | .179 | 114 |
| 11 | 2 | 3.1 | .145 | 32.2 |
| 12 | 2 | 2.0 | .033 | 11 |
| 13 | 2 | 1.1 | .028 | 16.4 |
| 14 | 2 | 2.5 | .12 | 32 |
| 15 | 2 | 6.0 | .315 | 35 |
| 16 | 2 | 1.7 | .163 | 63.5 |
| 17 | 2 | 2.7 | .075 | 18.5 |
| 18 | 0.75 | 1.3 | .021 | 10.8 |
| 19 | 2 | 1.5 | .08 | 34.2 |
| 20 | 2 | 1.75 | .078 | 29.9 |
| 21 | 2.1 | 3.4 | .069 | 13.5 |
| 22 | 2 | 0.48 | .066 | 78.6 |
| 23 | 2.1 | 2.2 | .078 | 23.7 |
| 24 | 1 | 1.5 | .091 | 39.4 |
| 25 | 0.54 | 3.8 | .16 | 28.0 |
| 26 | 2.2 | 1.9 | .039 | 14 |
| 27 | 0.9 | 2.8 | .023 | 5.5 |
| 28 | 2 | 2.5 | .165 | 44 |
| 29 | 2 | 1.2 | .151 | 83.8 |
| 30 | 2 | 2.6 | .036 | 9.2 |
| 31 | 2 | 0.8 | .034 | 27.0 |
| 32 | 2 | 1.6 | .053 | 22.1 |
| 33 | 2 | 3.2 | .091 | 19 |
| 34 | 2 | 2.3 | .055 | 15.9 |
| 35 | 2 | 2.5 | .045 | 11.8 |
| 36 | 2 | 0.6 | .08 | 88.8 |
| 37 | 2 | 1.8 | .038 | 14.4 |
| 38 | 1.3 | 4.3 | .371 | 58.1 |
| 39 | 2 | 1.9 | .044 | 15.4 |
| 40 | 2 | 0.7 | .024 | 22.2 |
| 41 | 2 | 3.1 | .08 | 17.2 |
| 42 | 1.8 | 0.9 | .047 | 36.4 |
| 43 | 2 | 1.5 | .015 | 6.7 |
| 44 | 0.7 | 2.4 | .090 | 25 |
| 45 | 2 | 3.2 | .272 | 56.7 |
| 46 | 1.6 | 1.4 | .046 | 21.9 |
| 47 | 2 | 4.7 | .122 | 17.3 |
| 48 | 1 | 0.7 | .038 | 36.2 |
| 49 | 2 | 2.2 | .067 | 20.3 |
| 50 | 2 | 1.4 | .106 | 50.5 |
| 51 | 2 | 1.5 | .093 | 41.3 |

Table B:2

Adipose tissue aromatase activity in women with benign breast disease

| No. | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | AROMATASE %CONV. | AROMATASE fmol/mg prot /hour |
|---|---|---|---|---|
| 1 | 0.53 | 1.93 | .057 | 19.8 |
| 2 | 1.75 | 1.1 | .095 | 58.6 |
| 3 | 0.83 | 2.3 | .126 | 41 |
| 4 | 0.6 | 4.7 | .054 | 7.7 |
| 5 | 1.9 | 1.7 | .061 | 26.3 |
| 6 | 1 | 2.7 | .062 | 15.3 |
| 7 | 2 | 2.5 | .055 | 14.7 |
| 8 | 0.7 | 3.2 | .067 | 14.1 |
| 9 | 2.2 | 4.4 | .055 | 8.3 |
| 10 | 0.9 | 3.4 | .055 | 10.8 |
| 11 | 1.5 | 3.1 | .101 | 23.9 |
| 12 | 2 | 2.8 | .038 | 9.0 |
| 13 | 1 | 2.4 | .074 | 20.6 |
| 14 | 0.7 | 1.4 | .057 | 27.5 |
| 15 | 0.6 | 1.4 | .027 | 12.9 |
| 16 | 2 | 2.5 | .032 | 8.5 |
| 17 | 2.2 | 4.8 | .052 | 7.2 |
| 18 | 2.2 | 3.8 | .058 | 10.2 |
| 19 | 2 | 1.5 | .098 | 43.6 |
| 20 | 0.6 | 3.6 | .095 | 19.4 |
| 21 | 1.2 | 2.9 | .113 | 27.5 |
| 22 | 0.7 | 3.8 | .027 | 4.7 |
| 23 | 0.5 | 2.1 | .014 | 4.4 |
| 24 | 1.5 | 2.7 | .07 | 17.3 |
| 25 | 2 | 1.2 | .028 | 15.6 |
| 26 | 1 | 5.0 | .083 | 11.1 |
| 27 | 2 | 2.3 | .024 | 7.0 |
| 28 | 0.5 | 3.1 | .029 | 6.2 |
| 29 | 2.2 | 3.2 | .050 | 10.4 |
| 30 | 1.5 | 1.5 | .053 | 22.9 |
| 31 | 1.1 | 1.9 | .069 | 24.5 |
| 32 | 0.7 | 3.1 | .044 | 10.3 |
| 33 | 2 | 1.3 | .006 | 3.1 |
| 34 | 2 | 2.6 | .022 | 5.6 |
| 35Bil | 2/2 | 3.3/4.3 | .046/.076 | 9.3/11.8 |

Table B:3.

17BHSD ACTIVITY IN BREAST ADIPOSE TISSUE FROM BREAST CANCER PATIENTS.

| No. | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | 17BOHSD %CONV | 17BOHSD pmol/mg prot /hour |
|---|---|---|---|---|
| 1 | 1.1 | 1.7 | 9.08 | 3.5 |
| 3 | 1.1 | 1.2 | 3.93 | 2.1 |
| 4 | 2 | 1.2 | 3.5 | 1.9 |
| 6 | 2 | 2.2 | 13.8 | 4.2 |
| 8Bil | 0.9/1.2 | 3.5/2.2 | 7.4/4.7 | 1.4/1.4 |
| 9 | 2.1 | 1.3 | 7.0 | 3.6 |
| 10 | 2 | 1.1 | 1.4 | 0.9 |
| 11 | 2 | 3.1 | 0.31 | .07 |
| 12 | 2 | 2.0 | 4.34 | 1.4 |
| 13 | 2 | 1.1 | 2.3 | 0.9 |
| 14 | 2 | 2.5 | 1.4 | 0.5 |
| 15 | 2 | 3.7 | 3.0 | 0.5 |
| 16 | 2 | 1.7 | 2.1 | 0.8 |
| 17 | 2 | 2.7 | 4.1 | 1.0 |
| 18 | 0.75 | 1.3 | 1.8 | 0.9 |
| 19 | 2 | 1.5 | 2.9 | 1.3 |
| 20 | 2 | 1.75 | 5.95 | 1.9 |
| 22 | 2 | 0.48 | 38 | 52.8 |
| 23 | 2.1 | 2.2 | 13.5 | 4.1 |
| 24 | 1 | 1.5 | 0.07 | .03 |
| 25 | 0.54 | 3.8 | 12.9 | 2.3 |
| 26 | 2.2 | 1.9 | 0.18 | 0.06 |
| 27 | 0.9 | 2.8 | 1.2 | 0.28 |
| 28 | 2 | 2.5 | 3.0 | 0.8 |
| 29 | 2 | 1.2 | 2.9 | 1.6 |
| 30 | 2 | 2.6 | 1.2 | 0.3 |
| 31 | 2 | 0.8 | 1.1 | 0.8 |
| 33 | 2 | 2.4 | 0.16 | .04 |
| 34 | 2 | 2.3 | 6.4 | 1.85 |
| 35 | 2 | 2.5 | 1.4 | 0.4 |
| 36 | 2 | 0.6 | 10 | 11.11 |
| 37 | 2 | 1.8 | 8.9 | 3.5 |
| 39 | 2 | 1.9 | 1.6 | 0.3 |
| 40 | 2 | 0.7 | 3.4 | 3.3 |
| 41 | 2 | 3.1 | 12.2 | 2.6 |
| 42 | 1.8 | 0.9 | 1.7 | 1.3 |
| 43 | 2 | 1.5 | 1.0 | 0.6 |
| 44 | 0.7 | 2.4 | 4.6 | 1.3 |
| 45 | 2 | 3.2 | 9.0 | 1.9 |
| 46 | 1.6 | 1.4 | 16.4 | 7.8 |
| 47 | 2 | 4.7 | 19.7 | 6.55 |
| 48 | 1 | 0.7 | 2.4 | 2.3 |
| 49 | 2 | 2.2 | 0.86 | 0.3 |
| 50 | 2 | 1.4 | 11.8 | 5.6 |
| 51 | 2 | 1.5 | 9.2 | 4.1 |

Table B:4

17BHSD activity in breast adipose tissue from women with benign breast conditions

| No. | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | 17BHSD %CONV | 17BHSD pmol/mg prot /hour |
|---|---|---|---|---|
| 1 | 0.53 | 1.93 | 1.33 | 0.4 |
| 2 | 1.75 | 1.1 | 5.77 | 3.6 |
| 3 | 0.83 | 2.3 | 4.4 | 1.3 |
| 4 | 0.6 | 4.7 | 0.9 | 0.1 |
| 5 | 1.9 | 1.7 | 2.1 | 0.7 |
| 6 | 1 | 2.7 | 2.0 | 0.5 |
| 8 | 0.7 | 3.2 | 0.7 | 0.1 |
| 9 | 2.2 | 4.4 | 5.1 | 0.8 |
| 10 | 0.9 | 3.4 | 0.9 | 0.2 |
| 11 | 1.5 | 3.1 | 11.7 | 2.5 |
| 12 | 2 | 2.8 | 14.9 | 3.5 |
| 13 | 1 | 2.4 | 7.1 | 2.0 |
| 14 | 0.7 | 1.4 | 4.8 | 2.3 |
| 15 | 0.6 | 1.4 | 1.4 | 0.7 |
| 16 | 2 | 2.5 | 6.3 | 1.7 |
| 17 | 2.2 | 4.8 | 5.2 | 0.7 |
| 18 | 2.2 | 3.8 | 8.3 | 1.5 |
| 19 | 2 | 1.5 | 2.7 | 0.6 |
| 20 | 0.6 | 3.6 | 6.7 | 1.2 |
| 21 | 1.2 | 2.9 | 15.8 | 3.5 |
| 22 | 0.7 | 3.8 | 1.7 | 0.3 |
| 23 | 0.5 | 2.1 | 0.9 | 0.3 |
| 24 | 1.5 | 2.7 | 6.1 | 1.5 |
| 25 | 2 | 1.2 | 0.8 | 0.4 |
| 26 | 1 | 5.0 | 1.8 | 0.2 |
| 27 | 2 | 2.3 | 17.4 | 5.1 |
| 28 | 0.5 | 3.1 | 2.1 | 0.4 |
| 29 | 2.2 | 3.2 | 7.1 | 1.5 |
| 30 | 1.5 | 1.5 | 3.7 | 1.6 |
| 31 | 1.1 | 1.9 | 4.2 | 1.5 |
| 32 | 0.7 | 3.1 | 0.7 | 0.1 |
| 33 | 2 | 1.3 | 20.1 | 10.3 |

Table B:5.

Aromatase activity in adipose tissue from the four quadrants of individual breasts.

| No. | QUAD | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | AROMATASE %CONV. | AROMATASE fmol/mg prot /hour |
|---|---|---|---|---|---|
| 4 | UOQ | 2.1 | 2.1 | .030 | 9.5 |
| | UIQ | 2 | 1.5 | .014 | 6.2 |
| | LIQ* | 2 | 1.2 | .019 | 10.5 |
| | LOQ* | 2 | 1.6 | .036 | 15 |
| 9 | UOQ | 2.1 | 4.3 | .079 | 12.3 |
| | UIQ* | 2.1 | 1.3 | .067 | 34.9 |
| | LIQ | 2.1 | 2.1 | .023 | 7.7 |
| | LOQ | 2.1 | 2.8 | .030 | 7.1 |
| 11 | UOQ* | 2 | 3.1 | .145 | 32.2 |
| | UIQ | 2 | 2.7 | .101 | 24.9 |
| | LIQ | 2 | 2.3 | .079 | 22.9 |
| | LOQ | 2 | 2.8 | .115 | 27.4 |
| 14 | UOQ* | 2 | 2.5 | .12 | 32 |
| | UIQ | 2 | 1.3 | .046 | 23.6 |
| | LIQ | 2 | 1.92 | .075 | 26.0 |
| | LOQ | 2 | 2.2 | .079 | 23.9 |
| 15 | UOQ* | 1.3 | 2.7 | .099 | 17.8 |
| | UIQ | 2 | 4.2 | .066 | 10.5 |
| | LIQ | 2 | 5.6 | .126 | 15 |
| | LOQ* | 2 | 6.0 | .315 | 35 |
| 18 | UOQ* | 1.2 | 1.3 | .014 | 7.2 |
| | UIQ* | 0.75 | 1.3 | .021 | 10.8 |
| | LIQ | 0.5 | 3.7 | .020 | 3.6 |
| | LOQ | 0.9 | 1.5 | .008 | 3.6 |
| 23 | OUQ* | 2.1 | 2.2 | .078 | 23.7 |
| | UIQ | 2 | 2.1 | .033 | 10.5 |
| | LIQ | 2.1 | 1.5 | .011 | 4.9 |
| | LOQ | 2 | 2.2 | .034 | 10.3 |
| 26 | UOQ* | 2.2 | 1.9 | .039 | 14 |
| | UIQ | 2.2 | 3.0 | .055 | 12.2 |
| | LIQ | 2.2 | 1.3 | .011 | 5.6 |
| | LOQ | 2.2 | 1.4 | .015 | 7.1 |
| 33 | OUQ* | 2 | 1.9 | .038 | 13.1 |
| | UIQ* | 2 | 2.4 | .050 | 14 |
| | LIQ | 2 | 3.1 | .046 | 10 |
| | LOQ* | 2 | 3.2 | .091 | 19 |
| 34 | UOQ* | 2 | 2.3 | .055 | 15.9 |
| | UIQ | 2 | 1.7 | .021 | 8.2 |
| | LIQ | 2 | 1.9 | .031 | 10.9 |
| | LOQ | 2 | 1.8 | .014 | 5.2 |
| 39 | OUQ* | 2 | 1.9 | .044 | 15.4 |
| | UIQ | 1.3 | 4.4 | .064 | 9.7 |
| | LIQ | 2 | 4.1 | .076 | 12.3 |
| | LOQ | 1.2 | 3.6 | .068 | 12.5 |
| 47 | UOQ* | 2 | 4.7 | .122 | 17.3 |
| | UIQ | 2.1 | 2.9 | .027 | 6.2 |
| | LIQ | 2.1 | 2.9 | .051 | 11.7 |
| | LOQ | 2 | 2.3 | .047 | 14 |

Table B:6.

17BHSD activity in adipose tissue from breast quadrants.

| No. | QUAD | ADIPOSE TISSUE (GM) | PROTEIN CONC (mg/ml) | 17BHSD %CONV | 17BHSD pmol/mg prot /hour |
|---|---|---|---|---|---|
| 4 | UOQ | 2.1 | 2.1 | 5.4 | 1.7 |
| | UIQ | 2 | 1.5 | 3.4 | 1.5 |
| | LIQ* | 2 | 1.2 | 3.5 | 1.9 |
| | LOQ* | 2 | 1.6 | 3.8 | 1.6 |
| 9 | UOQ | 2.1 | 4.3 | 4.7 | 0.7 |
| | UIQ* | 2.1 | 1.3 | 7.0 | 3.6 |
| | LIQ | 2.1 | 2.1 | 5.7 | 1.9 |
| | LOQ | 2.1 | 2.8 | 4.9 | 1.2 |
| 11 | UOQ* | 2 | 3.1 | 0.31 | 0.067 |
| | UIQ | 2 | 2.7 | 0.28 | 0.069 |
| | LIQ | 2 | 2.3 | 0.19 | 0.055 |
| | LOQ | 2 | 2.8 | 0.28 | 0.067 |
| 14 | UOQ* | 2 | 2.5 | 1.4 | 0.5 |
| | UIQ | 2 | 1.3 | 1.4 | 0.47 |
| | LIQ | 2 | 1.92 | 1.7 | 0.57 |
| | LOQ | 2 | 2.2 | 2.8 | 0.9 |
| 15 | UOQ* | 1.3 | 2.7 | 3.0 | 0.5 |
| | UIQ | 2 | 4.2 | 3.1 | 0.5 |
| | LIQ | 2 | 5.6 | 3.6 | 0.4 |
| | LOQ* | 2 | 6.0 | 14.9 | 1.7 |
| 18 | UOQ* | 1.2 | 1.3 | 1.7 | 0.87 |
| | UIQ* | 0.75 | 1.3 | 1.8 | 0.9 |
| | LIQ | 0.5 | 3.7 | 3.1 | 0.56 |
| | LOQ | 0.9 | 1.5 | 1.6 | 0.7 |
| 23 | OUQ* | 2.1 | 2.2 | 13.5 | 4.10 |
| | UIQ | 2 | 2.1 | 18.3 | 5.8 |
| | LIQ | 2.1 | 1.5 | 9.3 | 4.13 |
| | LOQ | 2 | 2.2 | 13.8 | 4.18 |
| 26 | UOQ* | 2.2 | 1.9 | 0.18 | 0.06 |
| | UIQ | 2.2 | 3.0 | 0.14 | 0.03 |
| | LIQ | 2.2 | 1.3 | 0.13 | 0.07 |
| | LOQ | 2.2 | 1.4 | 0.17 | 0.08 |
| 33 | OUQ* | 2 | 1.9 | 0.19 | 0.065 |
| | UIQ* | 2 | 2.4 | 0.16 | 0.044 |
| | LIQ | 2 | 3.1 | 0.18 | 0.039 |
| | LOQ* | 2 | 3.2 | 0.22 | 0.046 |
| 34 | UOQ* | 2 | 2.3 | 6.4 | 1.85 |
| | UIQ | 2 | 1.7 | 5.6 | 2.2 |
| | LIQ | 2 | 1.9 | 5.8 | 2.0 |
| | LOQ | 2 | 1.8 | 10.0 | 3.7 |
| 39 | OUQ* | 2 | 1.9 | 1.6 | 0.3 |
| | UIQ | 1.3 | 4.4 | 2.4 | 0.36 |
| | LIQ | 2 | 4.1 | 2.3 | 0.37 |
| | LOQ | 1.2 | 3.6 | 2.1 | 0.39 |
| 47 | UOQ* | 2 | 4.7 | 19.7 | 6.55 |
| | UIQ | 2.1 | 2.9 | 7.3 | 2.3 |
| | LIQ | 2.1 | 2.9 | 13.5 | 4.3 |
| | LOO | 2 | 2.3 | 3.7 | 1.2 |