University of Southampton

Sulphur amino acid metabolism, oxidative stress and pancreatitis.

The research comprising this thesis was conceived and performed within The Pancreato-Biliary Service at The Royal Infirmary, Manchester, UK.

Dr. Nicholas M. Sharer BSc. BM. FRCP.

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ABSTRACT

FACULTY OF MEDICINE

Doctor of Medicine

SULPHUR AMINO ACID METABOLISM, OXIDATIVE STRESS AND PANCREATITIS

Dr Nicholas M. Sharer

Pancreatitis remains a potentially fatal disease for which no specific therapy is available because its pathogenesis is poorly understood. Evidence supports aberrant oxidative stress as the trigger which results in the misdirection of zymogen exocytosis from acinar cells and interruption of regulatory methylation reactions. The observation that mice fed a diet that blocks the recycling and metabolism of methionine develop fulminant pancreatitis, attests to the dependence of the pancreatic acinar cell on methionine transsulphuration.

S-adenosylmethionine (SAMe), the first intermediate of transsulphuration, is the principle metabolic methyl group donor. Glutathione (GSH) a final product of transsulphuration is a vital intracellular antioxidant. N-acetylcysteine (NAC), an antioxidant in its own right potentially bolsters GSH levels. Thus, the combination of SAMe and NAC could be expected to ameliorate and/or accelerate recovery from acute pancreatitis. However, intravenous SAMe and NAC for the first 24 hours of acute pancreatitis, proved to be of no advantage over optimal conventional care in a randomised trial. Low blood folate levels were identified as a potential explanation for treatment failure, as also was vitamin C.

Plasma methionine concentration was similar in healthy controls and patients with quiescent recurrent acute (RAP) or chronic (CP) pancreatitis. Oral loading with methionine or NAC exposed no differences between these groups in methionine pharmacokinetics, or the concentration of (i) amino acids whose metabolism is linked with methionine or (ii) transsulphuration intermediates, except for plasma GSH, which is low in pancreatitics. Serum selenium and vitamin C were low in patients with pancreatitis, as was urinary inorganic sulphate excretion in CP. These vitamin deficiencies are corrected within 10 weeks by oral antioxidant supplementation therapy [*Bioantox.* i q.i.d. *PharmaNord*] (AOT). AOT did not alter blood levels or pharmacokinetic characteristics of methionine, GSH or other amino acids measured.

Irrefutable evidence implicates oxidative stress and/or a disruption of methionine metabolism in the genesis of pancreatitis. Further studies to elucidate the mechanisms that initiate and prevent/reverse these metabolic perturbations should facilitate the development of an antioxidant cocktail with prophylactic and therapeutic activity against this condition.

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Dedication

For my father, Dr. Philip Sharer, to whom a while before he died, I'd suggested that I might follow a career as a Biochemist. Thirty years later - this is the closest I'll come.

Acknowledgments

The research described in this thesis represents part of an on-going programme in The Pancreato-Biliary Laboratories of Manchester Royal Infirmary. The studies were conceived by Dr Joan M. Braganza in 1986, as one aspect of a series of investigations into intermediate metabolism, that attempt to explain the aetiogenesis of pancreatitis. Her extraordinary ability to collate published observations from vastly diverse fields, which to the majority of us appear to be as unrelated as the chicken and the egg; develop them into theoretical models based upon fact and then to devise practical approaches with which to test her hypotheses, is unique. As a consequence of her unequaled ability she is simply decades ahead of her time. To work closely with has been annoying, awe-inspiring, frustrating, humbling, stimulating but more than anything has been an immense learning experience. Thank you.

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Penultimately, I will be forever in the debt of the patients who went through the Pancreatic Services of The Royal Infirmary during the time I was in the department. It is from them, because of them and with them that my knowledge and interest in Pancreatology has grown. Individually and jointly we encountered a range of emotions and experiences. The sixteen challenge study volenteers (and the eight controls) became friends and their special qualities will remain with me forever, as will memories of several hundreds of other patients with pancreatitis, some of who did not survive their disease or who are no longer with us.

Finally, to my wife Debbie, our children Naomi, Leo and Toby, my family and friends, who have endured the demands that this thesis placed on them.

Abbreviations

Abbreviated form	*Full form*
6PG	6-Phosphogluconate
AOT	Antioxidant supplementation therapy
AP	Acute pancreatitis
APACHE II	Acute physiology and chronic health evaluation II scoring system
ATP	Adenosine triphosphate
AUC	Area under curve
BSE	Bovine spongiform encephalitis
χ^2	Chi Squared test
CDE	Choline-deficient ethionine supplemented diet
CP	Chronic pancreatitis
FROPs	Free radical oxidation products
G6P	Glucose-6-phosphate
GSH	Glutathione (reduced)
GSSH	Glutathione (oxidised)
GSH-Px	Glutathione peroxidase
GSH-Rx	Glutathione reductase
GSH-Tr	Glutathione transferase
GTP	Guanosine 5'-triphosphate
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
LDL	Low density lipoprotein
LPS	Lipopolysaccaride (endotoxin)
MDA	Malondealdehyde
MEOH	Methanol
MSOF	Multi-system organ failure
MCR	Metabolic clearance rate

NAC	N-acetylcysteine
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NSF	N-ethylmaleimide sensitive factor
OH^*	Hydroxyl radical
OTC	L-2-oxothiazolodine-4-carboxylic acid
p	Probability
PPi	Pyrophosphate
ppm	parts per million
RAP	Recurrent acute pancreatitis
RCT	Randomised controlled trial
RH	Saturated fatty acid
ROH	Oxidation product of fatty acid
s.d.	Standard deviation
SAA(s)	Sulphur amino acid(s)
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
s.e.m.	Standard error of the mean
SOD	Superoxide dismutase
SO_4^{2-}	Sulphate ion
SSA	Sulphosalicylic acid
t½	Half life
THF	Tetrahydrofolate
TNF-alpha	Tumour necrosis factor alpha (α)
Vd	Volume of distribution

Chapter 1

Sulphur Amino Acid Metabolism, Oxidative Stress and Pancreatitis

1.1.1. Introduction to the Manchester Philosophy

The Manchester Model, for the pathogenesis of pancreatitis accommodates the diverse aetiological factors and overlapping clinicopathological features of 'acute' and 'chronic' pancreatitis (Braganza 1983, 90, 91, 96 and 98). Briefly, it proposes that oxidative stress, by interfering with the polarised exocytosis of zymogens from the pancreatic acinar cell, results in an interstitial as well as in some cases a systemic inflammatory reaction. Oxidative stress from an excess of free radical activity over antioxidant availability may result because of:

- an absolute increase in free radical load as a result of:
  - ⇒ induction of the cytochrome P450 'detoxification' systems (Haboubi 1986, Acheson 1989, Sandilands 1990, Foster 1993, Braganza 1993a)
  - ⇒ heightened production of the reactive oxygen intermediates $O_2^{-\bullet}$, H_2O_2 and $OH^{\bullet}$ (Nonaka 1989b, Fernández-Checa 1998, Wereszczynska-Siemiatkowska 1998)
  - ⇒ Conversion of xanthine dehydrogenase to the $O_2^{-\bullet}$ producing xanthine oxidase (XO) (Sanfey 1985, Braganza 1991).
- an absolute dietary lack of the micronutrients required to sustain glutathione and other antioxidant defences (Märtensson 1986b, Schofield 1991, Segal 1995).
- a relative imbalance in favour of free radicals, although their production rates and antioxidant levels are still 'normal' (Uden 1988b).

Figure 1.1 schematises these various free radical producing and antioxidant quenching mechanisms.

Whereas the cytochrome P450 oxygenase enzymes are distributed widely in many organs, the contribution of the extra-hepatic pool to overall xenobiotic disposition is small and xenobiotic detoxification is predominantly hepatic (Braganza 1993a).

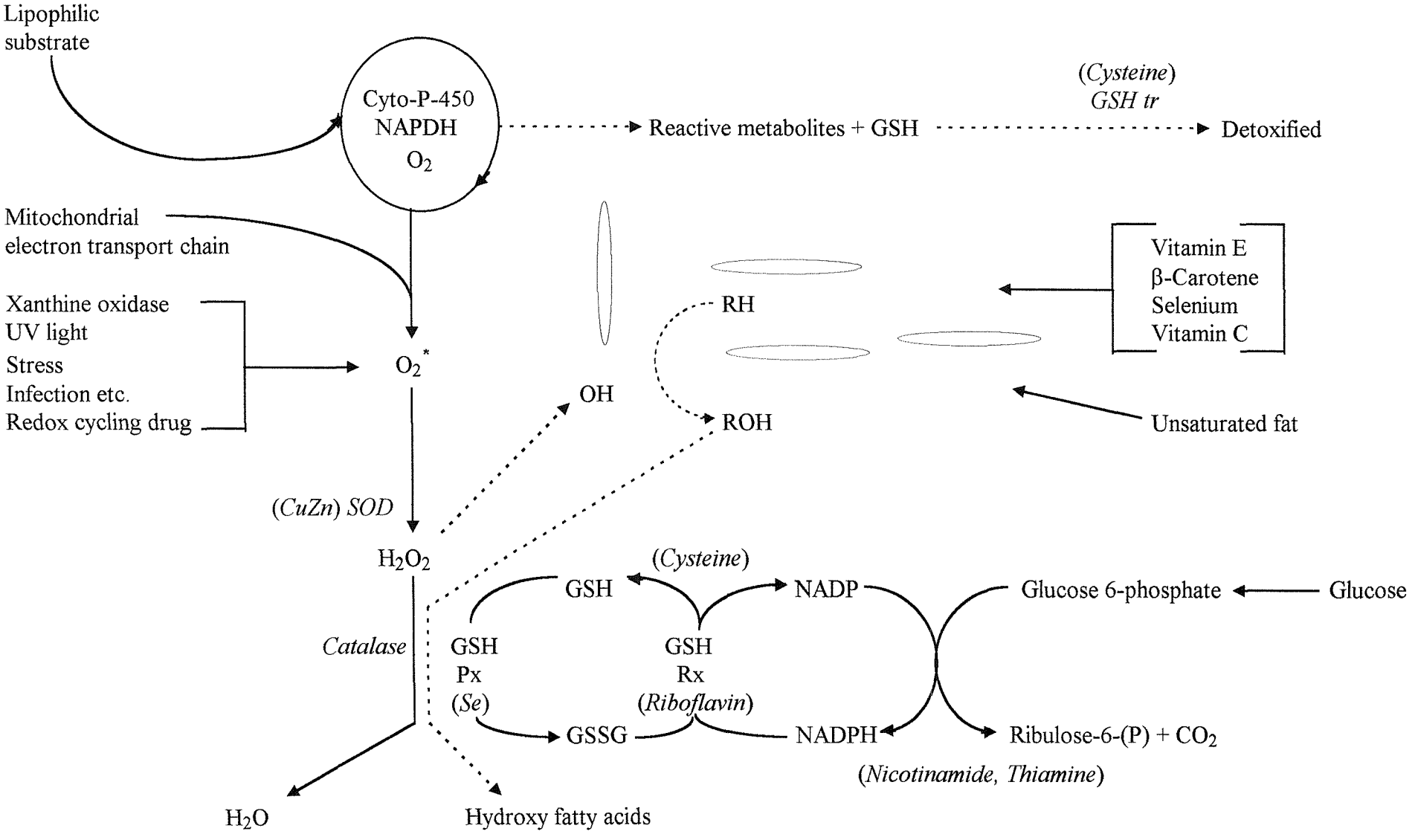


Figure 1.1 **Generators and quenchers of reactive oxygen species and drug metabolites in cells**

Key to Figure 1.1. Generators and quenchers of reactive oxygen species and drug metabolites in cells. SOD - Superoxide dismutase; RH - Saturated fatty acid; ROH - Oxidation product of fatty acid; GSH - Reduced glutathione; GSSG - Oxidised glutathione; GSH-Px - Glutathione peroxidase; GSH-Rx - Glutathione reductase; GSH-Tr - Glutathione transferase; NADP(H) - Nicotinamide adenine dinucleotide phosphate (reduced); Cyto 450 - Cytochrome P 450. Essential nutrients for different functions are indicated in italics in parentheses. *After* ***Braganza 1991***.

Cytochrome P450 enzymes are readily induced in the liver and similarly have been found to be induced within the pancreas in both pancreatitis and pancreatic cancer (Uden 1988a, Acheson 1989, Foster 1993).

Oxidative stress in the locality or within acinar cells is associated with a compensatory reversal of their secretory polarity, that results in the release of zymogens, pro-inflammatory free radicals and their oxidative products into the pancreatic interstitium (Braganza 1991 & 95b). A local inflammatory reaction results in the attraction of hordes of polymorphonuclear leukocytes into the pancreas, with the development of the clinical entity of acute pancreatitis (AP). In the majority of cases, local and systemic anti-inflammatory and anti-protease defenses contain this inflammatory response (Braganza 1995b). Should the stimulus be overwhelming and / or prolonged, it may escape normal regulatory control with the subsequent and often rapid development of the much feared and frequently fatal multisystem organ failure syndrome (MSOF) (Braganza 1996).

After resolution of an acute attack, if antioxidant availability fails to keep pace with oxidant load, then a persistent 'low-grade' inflammatory response from continued misdirection of zymogens into the interstitium may ensue - a potential mechanism for transition from 'acute' to 'chronic' pancreatitis (Ammann 1994, Riaz 1997). The clinical symptoms encountered and the 'aggressiveness' of the histological response being dependant upon the balance between the pro- and anti- inflammatory arms of the initiating, perpetuating and inhibiting elements contributing to oxidative stress (Braganza 1996).

Events such as these would explain the increased interstitial gland pressure, the creeping fibrosis that strangles ducts producing ductal hypertension and the excitation of nociceptive nerve endings, with the characteristic morphological and symptomatic features of chronic pancreatitis (CP). Furthermore, secretion of local antioxidants increases and the precipitation of some of these, such as lactoferrin and mucin may underlie pancreatic stone formation (Braganza 1996, 98).

The corollary that antioxidant supplementation should prevent further attacks was demonstrated in a double blind placebo-controlled cross-over study (Uden 1990a) and supported by two further trials (Bilton 1994) and in long term observations (Whitely 1994, McCloy 1998). The latter suggested that antioxidant therapy could render redundant extensive pancreatic surgery with its attendant peri-operative and long-term morbidity and mortality.

Glutathione (GSH) is probably the most important intra-cellular antioxidant. Although the exocrine pancreas is now known to function at physiological levels of low-grade oxidative stress, it is also exquisitely sensitive to unfettered free radical activity above this limit (Wallig 1998). Supplies to meet the physiological demands for GSH, necessitate integrity of the methionine transsulphuration pathway (Figure 1.2). Disruption of this pathway affects intracellular energy charge and membrane fluidity by impairing phospholipid methylation, because the concentration of the universal methyl donor, S-adenosylmethionine (SAMe) falls (Finkelstein 1978). Recycling homocysteine to methionine is one mechanism by which SAMe availability is bolstered, but homocysteine used in this manner is not available for subsequent onward metabolism to glutathione (Finkelstein 1984). This manifests in a number of organs, none more so than in the pancreas. Integrity of the transsulphuration pathway is vital for maintaining regulated pancreatic exocrine function (Goldberg 1950, Véghelyi 1962, Müller-Wieland 1964b), while its disruption, as in the inhibition of GSH synthesis by L-buthionine-[S,R]-sulfoxime precipitates pancreatitis (Lüthen 1994c).

For evidence that sulphur amino acid metabolism is intimately involved with pancreatitis, we need look no further than the lethal experimental model of the disease produced by the methionine analogue, ethionine (Faber 1950, Goldberg 1950 & 1951, De Almeida 1952, Müller-Wieland 1964b, Lombardi 1975 & 1976). Furthermore, oxidative stress inhibits several enzymes of the methionine transsulphuration pathway (Davies 1986) and pertubations of methionine and its transsulphuration products have been reported at admission with an attack of pancreatitis (Mårtensson 1986a, Roth 1985, Schofield 1991). The pathogenesis of chronic liver diseases, alcoholic cirrhosis in particular, has much in common with CP.

Impairment of methionine metabolism and the transsulphuration pathway in cirrhosis is recognised (Kinsell 1947, Horowitz 1981). The potential for GSH production / reconstitution in the hepatocyte is orders of magnitude greater than the pancreatic acinar cell: the corollary is that the potential for damage from reactive intermediates e.g. from carbon tetrachloride if far higher in the acinar cell than the hepatocyte (Véghelyi 1962).

It was on this background that these studies were devised to further investigate sulphur amino acid metabolism in the context of pancreatic disease.

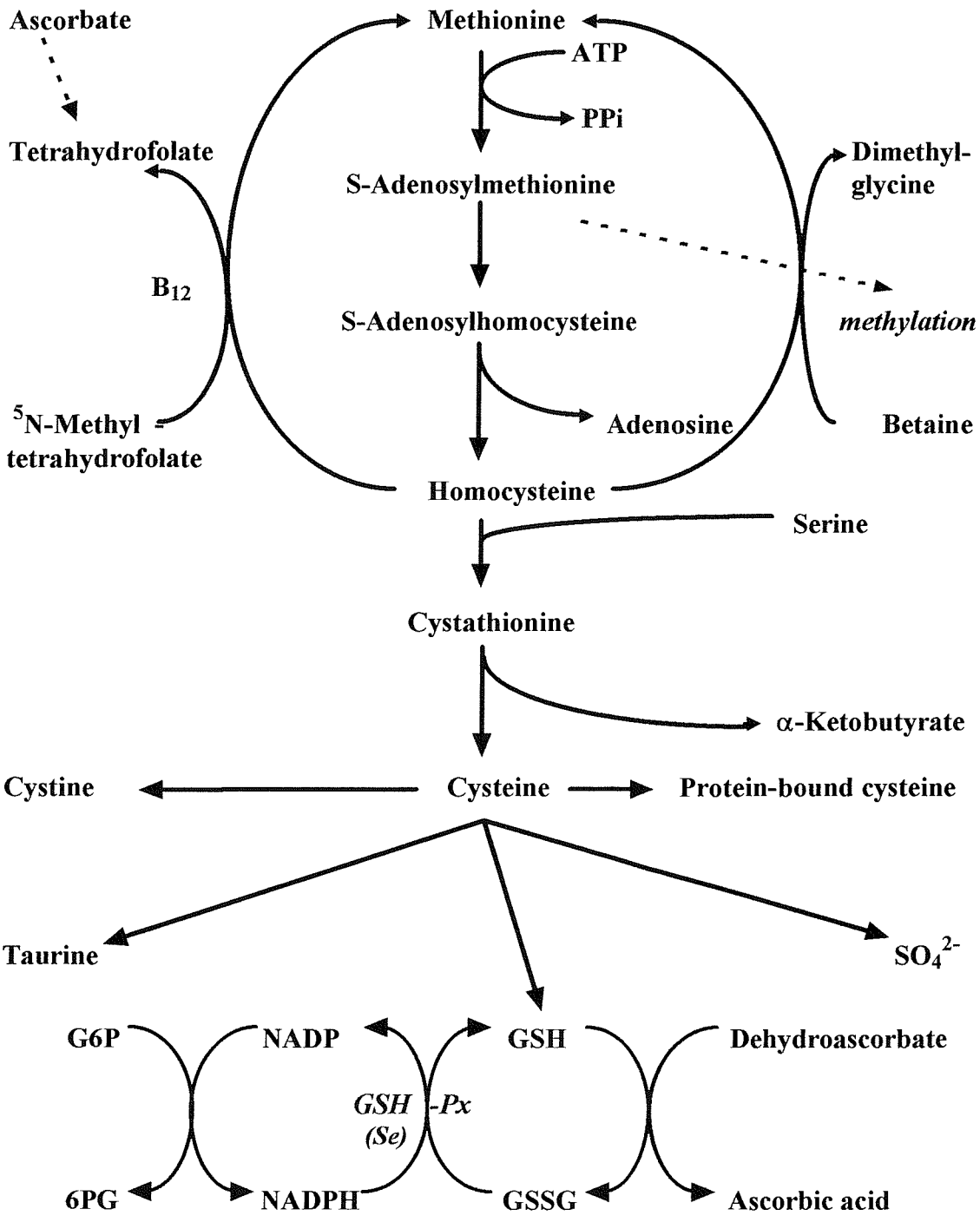


Figure 1.2. **The Methionine Transsulphuration Pathway**

1.2.0. Background to concept components

1.2.1 Oxidative stress

Defined as "the potential for tissue injury from an imbalance between free radical production, on the one hand, and available antioxidant defences on the other," oxidative stress underlies the onset / initiation of pancreatitis (Sanfey 1985, Wisner 1988, Nonaka 1989a, Guyan 1990, Braganza 1991 & 1998). The initial strands of evidence to support this connection in man came when analysis of duodenal juice and blood in patients with pancreatitis revealed augmented levels of free radical oxidation products (Braganza 1983, Uden 1988b), despite high levels of the natural antioxidants lactoferrin (Balakrishnan 1988) and ferroxidase II (Bell 1981) in these fluids, respectively. Free radicals, especially the hydroxyl radical ($OH^{\bullet}$), have been implicated (Nonaka 1989a). Dabrowski (1990) reports of increased free radical-mediated lipid peroxidation production in the early phases of experimental oedematous pancreatitis. Caerulein-induced AP results in upregulation of the oxidative-stress responsive genes as evidenced by rapid increase in mRNAs of c-fos, heme oxygenase-1, metallothionein-I and the cytokines IL-6 and TNF-alpha (Fu 1997 & 1998).

The first question needing an answer, is what is the origin of oxidative stress in the context of pancreatitis. Any lines of investigation that attempt to explain this should take into account that although the disease has many differing aetiologies, they result in a clinical condition that is physically, biochemically, radiologically and even prognostically indistinguishable. Free radicals originate from a number of sources;

i) the oxidative phosphorylation electron transport chain situated within the mitochondria,
ii) xanthine oxidase activated under conditions of ischaemia and
iii) the oxidative detoxification of xenobiotics.

Flux through the electron transport chain is tightly regulated *in vivo*. Only under the unusual circumstances of hypoxia / reperfusion injury is an excess of oxygen radicals

generated. It is probably this mechanism that is involved after cardiac bypass procedures and in pancreatic grafts immediately post transplantation.

The xenobiotic detoxification 'route' involved permutations and combinations of three scenarios:

- *Chronic induction of pancreatic cytochrome P450 by the sum of xenobiotic exposure.* This is supported by pharmacokinetic studies (Acheson 1989) that demonstrated increased Theophylline and Antipyrine clearance in patients with pancreatic disease. Further evidence is available in the form of immuno-histochemistry. Foster (1993) confirmed induction of a number of cytochrome P450 oxido-reductases in the pancreas of patients suffering both chronic pancreatitis and pancreatic cancer. These studies have now been confirmed by other groups (Norton 1997, Wacke 1998).

- *Concurrent exposure to a chemical that undergoes bio-inactivation,* e.g. halogenated / aliphatic hydrocarbons in the occupational environment. McNamee (1994) identified occupational exposure to high levels of inhaled hydrocarbons as a positive risk factor for the development of chronic pancreatitis, confirming prior observations (Braganza 1986a). Furthermore, the Manchester group has reported exposure to petrochemical derivatives in patients in whom their attacks of pancreatitis were reduced or controlled by antioxidant supplements (Sandilands 1990).

- *A lack, either absolute or relative to increased demand, of micronutrient antioxidants or antioxidant precursors.* This factor may be the most important of the three. For example, patients suffering from epilepsy, often have enzyme induction stimulated by their long-term anticonvulsant therapy. In a study comparing patients suffering from epilepsy and pancreatitis with age / gender matched controls, discriminant analysis of pharmacokinetic and dietary data picked out lower intakes of methionine, vitamin C and to a lesser extent selenium, as the main determinants of patients with CP from these groups (Rose 1986, Uden

1988a). No association is recognised between anticonvulsant therapy and pancreatitis. An explanation for this would seem to be, that the epilepsy sufferers, who out of necessity lived in a sheltered environment, had their dietary intakes guaranteed.

1.2.2 Methionine transsulphuration

Methionine, considered an essential amino-acid in man, lies at the start of the transsulphuration pathway (Figure 1.2). Evidence of poor methionine intake in patients with chronic pancreatitis (Rose 1986) and of disturbed methionine metabolism at admission with an alcohol-induced attack of acute pancreatitis (Mårtensson 1986a, Roth 1985), reinforces the need to unravel how transsulphuration is regulated and disruption of it plays a role in the initiation of AP, recurrent acute pancreatitis (RAP) and CP.

Integrity of the methionine transsulphuration metabolic pathway (Figures 1.2 and 1.3) is central not just to pancreatic function, but also to the biochemical homeostasis of the whole organism (Finkelstein 1978). The pathway links sulphur amino-acid availability, metabolism and transfer of one-carbon units, control of intra-cellular energy charge and the trans-methylation processes that alter membrane fluidity and provide much of the regulatory secondary messenger systems. Disturbances to, or interruption of this pathway have been identified in as diverse clinical entities as accelerated atherosclerosis (Blom 1988, Clarke 1991, & 1998, Perry 1995), cerebral atrophy (Bleich 2000b), megaloblastic anaemia (Chanarin 1992, Hoffbrand 1993), nephrolithiasis (Broadus 1979), neural tube defects (Berry 1999) and in the context of this thesis, pancreatitis (Goldberg 1950, Roth 1985, Mårtensson 1986a, Schofield 1991, Lüthen 1994a).

1.2.3. Methionine Depletion in Pancreatitis

Initial evidence that methionine might be essential for the pancreas came from the elegant studies of Véghelyi half a century ago (Véghelyi 1950). He devised a diet

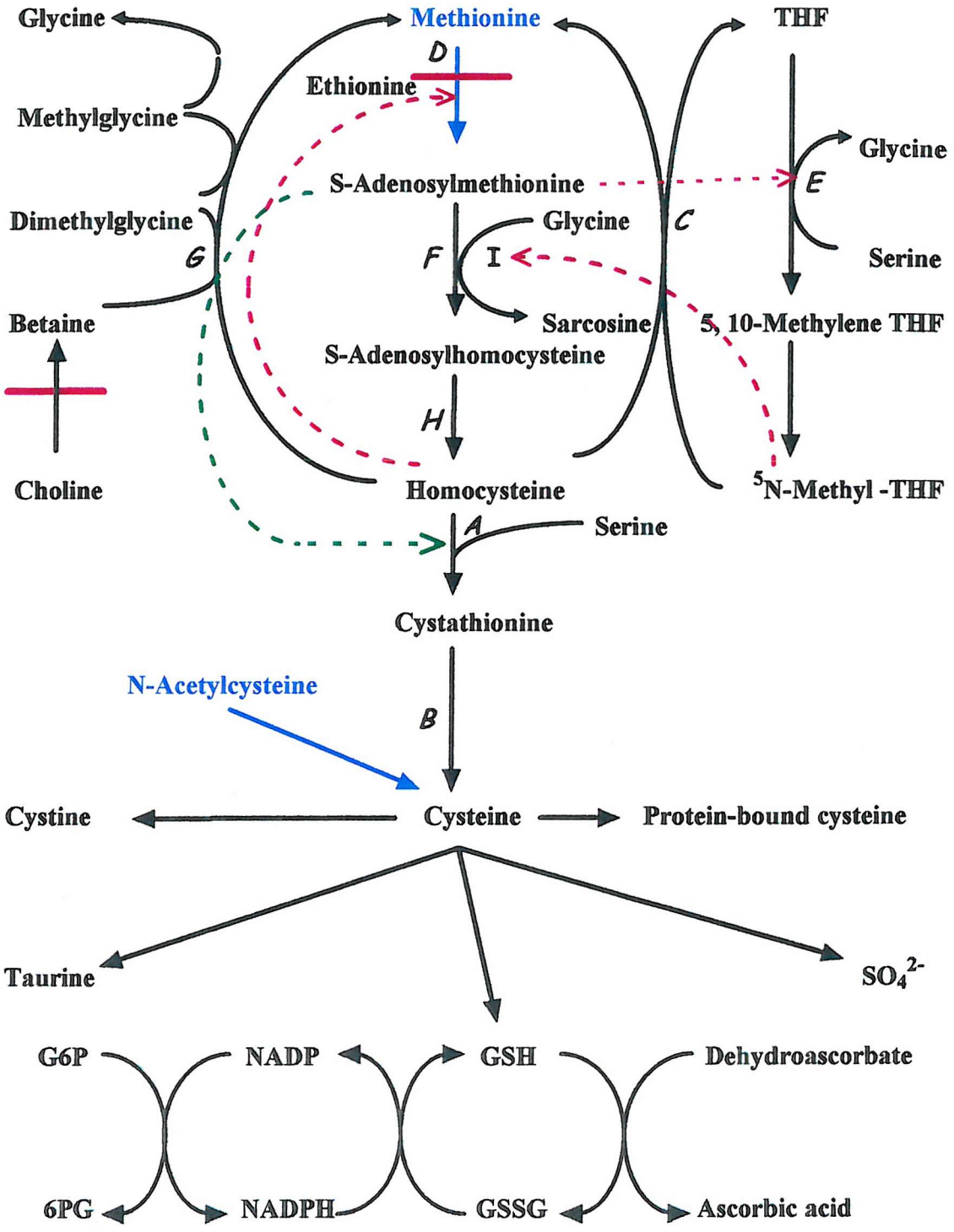


Figure 1.3. Augmentation, regulation and modulation sites of the methionine transsulphuration pathway

Key to Figure 1.3.

Enzymes:

A. Cystathionine β-synthetase
B. Cystathionase γ-lyase
C. Methionine synthetase
D. S-Adenosylmethionine synthetase
E. Serine hydroxymethyltransferase
F. Homocysteine methyltransferase (various methyl acceptors)
G. Betaine-homocysteine methyltransferase
H. Adenosylhomocysteinase
I. Glycine N-methyltransferase

Red bars (solid) ——— Sites where transsulphuration pathway is interrupted by the CDE diet, that results in potentially fatal experimental haemorrhagic pancreatitis.

Red arrows (broken) - - - - - Negative allosteric feedback inhibition of enzyme by metabolic intermediate.

Green arrows (broken) - - - - - Positive allosteric feedback inhibition of enzyme by metabolic intermediate.

Blue compounds / arrows ——— Sites at which challenge compounds feed into transsulphuration pathway.

Abbreviations:

THF	tetrahydrofolate
G6P	glucose 6 phosphate
6PG	6 phosphogluconate
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
GSH	glutathione
GSSG	oxidised glutathione
SO_4^{2-}	inorganic sulphate

that supplied 10 percent of the methionine and 50 percent of the tryptophan requirements. Feeding this diet to rats released the following chain of events within acinar cells: disappearance of the zymogen granules from the supranuclear zone, acidophilia and then vacuolisation of the cytoplasm, cyst formation and finally scarring strangulating the acini. Putting tryptophan back into the diet had no effect, but addition of methionine wholly prevented the changes (Véghelyi 1962).

1.2.4. The Choline-Deficient Ethionine Supplemented Diet (CDE diet)

Ethionine, on the other hand, is a methionine analogue that competitively binds to S-adenosylmethionine synthetase, blocking the first enzymic step of methionine transsulphuration. In 1950 it was reported that a single intraperitoneal injection of ethionine resulted in pancreatic cell vacuolation and necrosis in rats (Farber 1950). This toxic effect of ethionine was reported concurrently by Goldberg, but these lesions were entirely prevented by the simultaneous injection of methionine (Farber 1950, Goldberg 1950 & 51). Interestingly these workers also reported the effects of a high fat / low protein diet on acinar cell morphology, a diet now recognised to promote pancreatic cancer (Braganza 1993a). Ethionine-related injury is enhanced if administered to animals previously maintained on a protein depleted feed regime, suggesting that the toxic effect is mediated through interference with protein metabolism (Wachstein 1951). Even at this early stage in unraveling the mechanism(s) underlying pancreatitis, it was reported that a blockade to the apical secretion of zymogens occurred in ethionine-induced disease (Kahn 1959).

In 1975 Lombardi working with Estes and Longnecker described a dietary model of acute haemorrhagic pancreatic necrosis (Lombardi 1975). Young female mice given a **c**holine-**d**eficient dietary feed supplemented at the 0.5% level with **e**thionine (**CDE** diet), developed an acute haemorrhagic pancreatitis with widespread necrosis of intra-abdominal fat tissues. This CDE diet remains the most reproducibly lethal experimental model of haemorrhagic pancreatitis. With it, 100% of mice die by day five from the systemic effects of shock associated with acute haemorrhagic pancreatitis. This is in direct opposition to the disease that is caused by ethionine

alone, in which, the animals usually survive destruction of over 95% of the exocrine pancreas and concomitant hepatic steatosis (Lombardi 1975). If 0.5% ethionine is added to a choline-supplemented diet then the resulting mortality rate is 10% rather than 100%. Thus choline lack compounds and renders lethal the effect of methionine deprivation.

1.2.5. The homocysteine to methionine recycling pathways.

The dual-pronged attack of the CDE diet is rationalised by reference to the red bars in Figure 1.3. Ethionine acts as a competitive inhibitor of SAMe production and choline deficiency limits the capacity of the pathway to regenerate methionine from homocysteine, via the choline-betaine shunt.

Isolated choline-deficiency is documented to cause significant changes in hepatocellular morphology. Similar changes would be expected in the pancreas, for both organs have major biosynthetic roles and respond in a comparable manner to a metabolic insult. Depletion of the hepatic methyl donor pool is associated with abnormal liver function tests and hepatic steatosis, demonstrable on abdominal computed tomography (Chawla 1989, Buchman 1995). Steatosis is recognised to develop when intracellular energy charge is depleted and / or the regulatory trans-methylation reactions are disrupted. Such perturbations impede the transport mechanisms that export lipoproteins and other metabolites from hepatocytes. For example, supplementation of total parenteral nutrition (TPN) with choline reverses TPN-associated hepatic steatosis (Buchman 1995). Analogous metabolic sequelae surely occur in pancreatic acinar cells when disruption of transmethylation results in dysfunction of the stimulus-secretion coupling process.

Support for such a theory is conferred by studies on choline requirements in ethanolic liver injury, which is not due to an increased caloric intake, but mediated by choline oxidation mechanisms (Best 1949). Given the propensity of chronic alcohol ingestion to cause acute and / or chronic pancreatitis such reports are of relevance to pancreatic disease. Choline oxidation produces betaine, the methyl

group donor for one of the two reactions by which homocysteine may be converted back to methionine. Supplementation of betaine ameliorates alcoholic steatosis (Barak 1997) and betaine methyltransferase activity is induced after methionine synthetase inhibition (Lumb 1983). Chronic alcohol ingestion by rats results in hepatic betaine depletion and the trapping of N^5-methyltetrahydrofolate (Barak 1985). N^5-methyltetrahydrofolate is a cofactor for the other enzyme responsible for methionine regeneration from homocysteine, namely, N^5-methyltetrahydro-folate-homocysteine methyltransferase (methionine synthetase). Whether such observations can be accounted for by the 'methyl-folate trap hypothesis (Hoffbrand 1993) over which doubt and conflicting evidence exists (Chanarin 1992), or whether alcohol adversely effects folate stores in other ways is unclear (Russell 1983). Feeding alcohol to rats submitted to jejunoileal bypass, reproduces the effect of alcohol-induced hepatic injury in man. In animals on whom this surgical procedure is performed, methionine supplementation has been found to reduce hepatic triglyceride accumulation, increase hepatic ATP content and improve histological changes (fatty infiltration). These benefits are partially antagonised by an alcohol intake of around 15g/day/kg body weight (Parlesak 1998).

1.2.6. Stimulus secretion coupling

The final step in stimulus-secretion coupling is the fusion of the secretory (zymogen) granule with the plasma membrane. Evidence supports a reversible methylation reaction as being necessary for the regulation of pancreatic exocrine secretion and G-proteins have been implicated in this role of signal-transduction and membrane trafficking (Capdevila 1997). It is thus of interest that glutathione depletion inhibits amylase release from isolated guinea-pig pancreatic acinar cells (Stenson 1983) and that the pancreas is not the only organ in which transsulphuration has been demonstrated as being necessary for exocytosis (Brown 1994). Thiol groups are required by GTP and Rab3 proteins in exocytosis (Lledo 1994) that involves the formation and maintenance of an exocytotic transmembrane pore (Monck 1994). Furthermore, reduction of glutathione by bromobenzene reduces the size of the hepatocyte extra-mitochondrial calcium pool and the

subsequent cytoskeletal changes responsible for the sort of surface bleb formation, that one would instinctively suggest would impede exocytosis (Jewell 1982).

Kurt Müller-Wieland working at Manchester Royal Infirmary reported histological changes of "loss of basophilia and reduced granularity of the apical cytoplasm of acinar cells; basement membrane fragmentation, and detachment of acinar cells from the basement membrane with subsequent necrosis," after intravenous doses of 1.33mg ethionine / g body weight in male rats (Müller-Wieland 1964b). He did not use female rats "because of the excessive fatty degeneration of the liver that occurs in the early stages of ethionine toxicity;" further evidence of the magnitude of disruption that can be caused when stimulus-secretion uncoupling occurs through inhibition of the transsulphuration pathway.

1.2.7. Pancreatic Methionine Metabolism

The pancreas has a very high biosynthetic rate, requiring rapid and intense uptake of essential amino acids for protein and enzyme synthesis. This feature was harnessed by Syrota and co-workers who reported the potential of ^{11}C-L-methionine uptake as a way of visualising the pancreas. This method soon became a way of imaging the hitherto 'invisible' organ and provided the first non-invasive investigation capable of diagnosing chronic pancreatitis, in which uptake of labelled methionine is patchy and markedly reduced (Syrota 1981).

In 1985 Eric Roth and co-workers in Austria reported abnormalities of plasma concentrations of intermediates of methionine transsulphuration, in patients with acute haemorrhagic necrotising pancreatitis. The onset of isolated renal failure was associated with increased cystathionine levels, while elevation of both cystathionine and methionine was found with the onset of multi-system organ failure (MSOF). The concentration of free amino acids in skeletal muscle were also estimated in that study, and interestingly although methionine levels were unchanged taurine rose in acute pancreatitis (Roth 1985).

The next strand of evidence that sulphur amino acid metabolism is of importance to pancreatic integrity came only 12 months later from Sweden. In this study 6 patients with alcoholic chronic relapsing pancreatitis were compared to a group of patients undergoing cholecystectomy. Elevated levels of methionine were found, this time in leukocytes and the urine of patients with pancreatitis. Furthermore, reduced leukocyte taurine and glutathione concentrations and low urinary cystathionine excretion also supports the theory that a block in the conversion of methionine to cysteine occurs in pancreatitis, Figure 1.3 and Table 1.1 *(after Mårtensson 1986a)*. The disparity between rising muscle taurine levels (Roth 1985) yet falling leukocyte concentrations (Märtensson 1986a) seems to be rationalised by the observation that in the catabolic state muscle free amino acid profiles rapidly become abnormal with the most notable being a decrease in glutamine and a rise in taurine.

A recent report that chronic alcohol ingestion significantly reduces methionine influx across the brush border membrane of jejunal mucosa in the pregnant rat (Polache 1996) heightens further the importance of finding elevated leukocyte methionine levels in pancreatitis. Thus, rather than low levels caused by a combination of reduced absorptive mechanisms and impaired pre-morbid dietary intake, elevated levels are actually observed.

Caerulein hyperstimulation is a commonly utilised model of acute oedematous pancreatitis in the rat. Infusion of 7.5 μg / kg / hr for six hours causes hyperamylasaemia and interstitial oedema resulting in a doubling in weight of the gland (Dabrowski 1990). This group reported a fall of 70% in the total sulphydryl groups (essentially GSH) in pancreatic tissue in caerulein-induced pancreatitis, Table 1.2 *(after Dabrowski 1990)*.

S-Adenosylmethionine (SAMe) lies at the heart of the transsulphuration pathway. The ratio of SAMe to S-Adenosylhomocysteine (SAH) dictates the intracellular energy charge and is of crucial importance in the regulation of transmethylation reactions. The importance of SAMe to pancreatic integrity was reported in a rat

Table 1.1 Urinary excretion and leukocyte concentration of SAAs and Taurine (Mean +/- SEM) after ***Mårtensson 1986a***

		Elective Cholecystectomy Patients (days post-operative)			Chronic Relapsing Pncreatitis		
	Controls	**day 0**	**day 1**	**day 3**	**day 0**	**day 1**	**day 3**
Taurine							
Urine	419+/-69	292+/-47	1118+/-171‡¶	609+/-125	917+/-132‡§	1520+/-246‡	626+/-151
leukocyte†	19.0+/-0.11	17.6+/-0.56	ND#	ND	15.5+/-0.78‡§	ND	ND
Methionine							
urine *	12.6+/-1.0	12.4+/-0.76	9.66+/-2.02	7.32+/-1.42‡¶	22.1+/-4.50‡§	7.96+/-2.06‡¶	4.72+/-0.84‡¶§
leukocyte†	17+/-1.0	18+/-2.0	ND	ND	35+/-5.0‡§	ND	ND
Cyst(e)ine							
urine *	92.3+/-12.0	56.7+/-16.0	102+/-8.80¶	67.0+/-25.5	132+/-23.4§	130+/-31.2	84.7+/-16.7¶
Non-sulphate sulphur							
urine *	13.3+/-1.80	12.9+/-0.85	14.9+/-3.83	15.5+/-3.43	24.3+/-4.04‡§	25.6+/-9.25	11.8+/-2.39¶

* μmol /m^2 body surface area/ 24h. † nmol /10^6 (taurine) and 10^9 cells (methionine). # not determined. ‡ statistically significant with respect to control group. ¶ statistically significant with respect to cholecystectomised subjects. § statistically significant with respect to pretreatment group.

Table 1.2 Malondialdehyde concentration [MDA], total content of sulphydryl compounds and distribution of sulphydryl compounds in the protein and non-protein fraction of pancreatic tissue in rats with Caerulein-induced AP (Mean +/- SEM) after ***Dabrowski 1990***.

Group	[MDA] nmol/mg protein	Sulphydryl compounds nmol/mg protein	Total sulphydryl compounds (percent)	
			protein	non-protein
Controls: 6 hr saline infusion (n=8)	0.111 +/- 0.009	46.6 +/- 8.4	59.64 +/- 11.96	40.36 +/- 11.96
6 hr caerulin infusion (n=10)	0.188 +/- 0.0129§	14.2 +/- 6.2§	29.7 +/- 5.02§	70.3 +/- 5.02§

§ statistically significant $p < 0.001$ compared to control animals.

transplantation pancreatitis model, in which rejection was prevented by cyclosporin A, but fatal haemorrhagic pancreatitis still occurred unless 25 mg/kg SAMe was given eight hourly for 10 days post operatively (Scott 1992). Furthermore, folate deficiency also reverses the SAMe : SAH ratio, presumably by limiting the recycling of homocysteine to methionine, resulting in acute pancreatitis (Balaghi 1992a).

1.2.8. Antioxidants in pancreatic disease

Aggressive oxygen species (hydroxyl and superoxide radicals, hydrogen peroxide, peroxynitryl and singlet oxygen to name but a few) can all damage biochemical components. These include DNA, proteins, carbohydrates, lipids and micronutrients e.g. vitamins A, C, E, and folic acid. As a result of the threat posed, biology has developed a complex hierarchical defence strategy against free radical-mediated attack. These lines include:

1) Cellular compartmentation e.g. cytoplasm, mitochondria, lysosome.
2) Defence enzymes e.g. SOD, CAT, GSH-Px.
3) Non-essential antioxidants e.g. Albumin, uric acid and GSH.
4) Essential radical scavengers and quenchers e.g. Ascorbic acid, α-Tocopherol, β-Carotene.

Despite these multilevel defences, in many pathophysiological situations local endogenous antioxidant defences are exceeded and biochemical and tissue injury result. In such circumstances augmentation of the defences, with the administration of exogenous antioxidants may be advantageous. Supplementation of the defence mechanisms of categories 1) and 2) above are not without problem. Intracellular compartmentation is not amenable to manipulation and exogenous enzymes administration pose difficulties with the mode of delivery and antibody-mediated reactions, including anaphylaxis. As yet only limited experience has been gained of this approach in human, with recombinant SOD showing some (Land 1994) or no (Pollak 1993) benefit in the ischaemia-reperfusion injury associated with renal transplantation. Probably the greatest potential benefit in ameliorating pancreatitis by manipulating antioxidant enzymes comes from the inhibition of xanthine oxidase.

This enzyme is converted from the inactive dehydrogenase (D) to the active oxidase (O) form by ischaemia-induced proteolytic cleavage of the former, Figure 1.4.

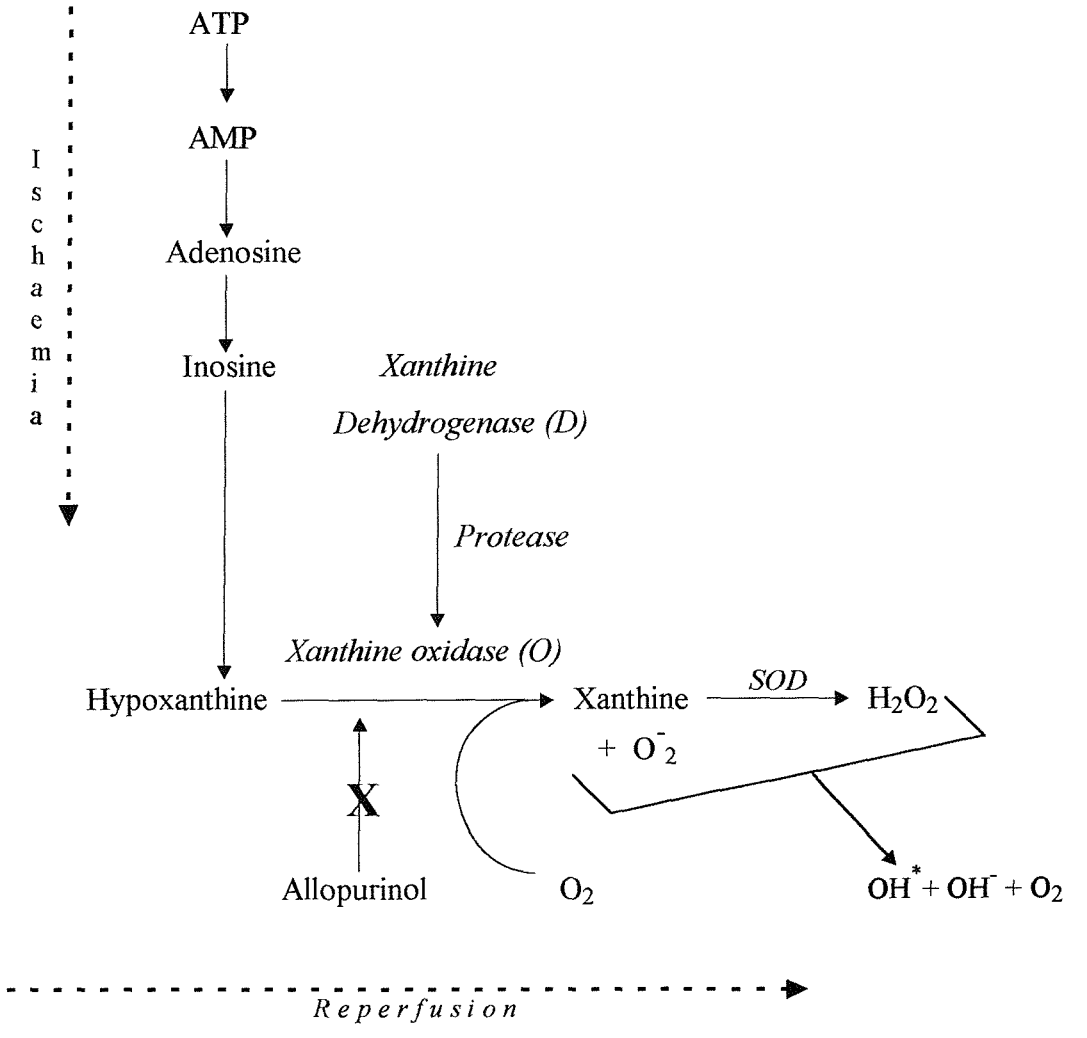


Figure 1.4. Mechanism for the production of oxygen free radicals in the presence of ischaemia, (after *Sanfey* in *Braganza 1991*).

Based on the above mechanism of oxygen radical production in the face of ischaemia, the corollary that Allopurinol treatment would be protective in experimental models of pancreatitis has been examined. In the free fatty acid infusion, partial duct obstruction with Caerulein hyperstimulation and the ischaemia-reperfusion models of experimental pancreatitis, pre-treatment with Allopurinol

ameliorated oedematous disease (Sanfey 1985). That this benefit has not been translated to humans is explained by the work of Sarr (1987) who confirmed the benefit of Allopurinol when given pretreatment. However, he also showed that the effect is reduced when the drug is given concurrent to induction of AP and lost when administered post-injury. Thus, administration in human disease could only be given pre-injury when given as prophylaxis before ERCP.

The group of antioxidant defences most amenable to modification are the essential micronutrients and vitamins. Derangement in the availability of a number of these elements and compounds have been implicated in a susceptibility to pancreatitis. Ascorbic acid, is the major aqueous phase antioxidant in human plasma. Levels have been reported to be low in admission samples of patients with acute pancreatitis (Scott 1993, Bonham 1999) and supplementation has been shown to ameliorate oedema in experimental pancreatitis (Nonaka 1991 & 1992). Vitamin B_6 is a co-factor for two enzymes of the methionine transsulphuration pathway (steps A and B in Figure 1.3.), and these are both vulnerable to oxidative stress-induced inhibition (Davies 1986). Oxygen radicals may damage folic acid, a vital co-factor in transsulphuration and methyl-group transfer. Diets lacking in folate inhibit amylase release and result in pancreatitis in rats (Balaghi 1993). Selenium, a co-factor of glutathione peroxidase (an enzyme vital to the recycling and replenishment of the major intra-cellular {non-essential} antioxidant - glutathione) has been reported to be depleted in acute and chronic pancreatitis (Schofield 1991). In chicks a selenium-depleted diet results in hyaline body formation in pancreatic acinar cells followed by cytoplasmic retraction towards the nucleus, and infiltration of fibroblasts and macrophages resulting in fibrotic change and death at 3 - 4 weeks of age. An observation that is completely prevented by selenium repletion (Gries 1972). Furthermore, selenium therapy has proven to reduce morbidity and mortality in human AP (Kuklinski 1991). The fat soluble vitamins act by chain breaking or scavenging and quenching free radicals, thereby limiting their deleterious effect. Examples of such are numerous and include α-tocopherol in ethanol-induced injury (Bondy 1996) and combinations containing Vitamins A

(including β-carotene) and E in preventing LDL-induced oxidative toxicity of islet beta-cells (Cnop 2002) are but two examples.

Singly, the essential antioxidants have been shown to exert a beneficial or protective role on cellular survival. Thus, it might be anticipated that in combination their effect would be additive or even cumulative. A 'global' antioxidant cocktail comprising methionine, vitamins A, C and E with selenium was advocated and proved beneficial in a cross-over study (Uden 1990a) and has also proven to be of benefit in the long-term (McCloy 1998) in RAP and CP. Additional support for the efficacy of this global approach has been offered more recently in CP (De las Heras Castano 2000), hyperlipidaemia-related pancreatitis (Heaney 1999) and hereditary pancreatitis in children (Uomo 2001).

1.3.0. Objectives of thesis

From the data presented, support is drawn for the hypothesis that the metabolism of sulphur-amino acids (in particular methionine) and oxidative stress (from an imbalance between free radicals production and available antioxidants) are major players in the aetiology of pancreatitis, that warrant further investigation. The evidence summarised above raises several hitherto unaddressed clinical questions in relation to pancreatitis. The work described in this thesis sought to answer three of these:

1) Might the immediate parenteral delivery of N-acetylcysteine (NAC) and S-adenosylmethionine (SAMe) - to replenish pancreatic thiols and methyl groups, respectively, be sufficient to ameliorate an attack.
2) Given that Vitamin B_{12}, folic acid, one-carbon and methionine metabolism are intimately and inseparably linked, what is Vitamin B_{12} and folic acid status at the time of and between attacks of pancreatitis.
3) Considering the implication of a functional blockade in the methionine transsulphuration pathway during a clinical attack, could challenging with methionine or NAC help to identify this vulnerability and pinpoint the site of disruption in the quiescent phase between attacks, and finally might this blockade be overcome by antioxidant supplementation?

In the chapters that follow, investigations directed at exploring these aspects of intermediate metabolism and the potential benefit of eliminating oxidative stress in pancreatitis are described.

Chapter 2

Trial of parenteral NAC and SAMe in acute pancreatitis

2.1.0. Introduction

Over the past decade work at Manchester has been aimed at developing a first-line medical treatment for acute pancreatitis. This was centered on the principle, that oxidative stress-induced hindrance to exocytosis from pancreatic acinar cells, pancreastasis, is the seminal problem (Braganza 1991). It seemed by analogy with cholestasis from paracetamol toxicity that depletion of glutathione (GSH) might be integral to the exocytosis blockade in pancreatitis, and hence treatment with precursors of this intracellular antioxidant thiol should help (Miners 1984, Braganza 1991).

In 1986 the dramatic effect observed after the administration of *N*-acetylcysteine (NAC) as soon as shock lung and renal shutdown occurred in a patient who had undergone drainage of a pancreatic pseudocyst under general anaesthesia 10 hours earlier (Holmes 1986); prompted a randomised pilot study over the next 15 patients with multi-organ failure, admitted under the care of a single Nephrologist. The drug was infused intravenously over 56 hours at a total of half the dose recommended for paracetamol poisoning (Braganza 1986b). As with the initial patient, the trial patients also received intermittent haemodialysis, with optimal cardiorespiratory support in a critical care unit. Acute pancreatitis was the precipitant in 6 patients, including 3 in the subgroup of 8 on optimal standard care (control arm) and 3 in the subgroup who also received NAC. Seven of the eight patients in the control arm of the study died, conforming with the expected mortality of 84% when the admission APACHE II scores (Knaus 1985) exceed 35, but there was only one death in the subgroup receiving NAC, although the admission APACHE II scores were similar (Figure 2.1). The dose of NAC was 300 mg/kg for the first day, half this for the next 2 days then half this dose again for the following and subsequent days.

By 1990 a placebo controlled trial had confirmed the value of oral antioxidants supplements in preventing recurrences of acute or acute-on-chronic pancreatitis

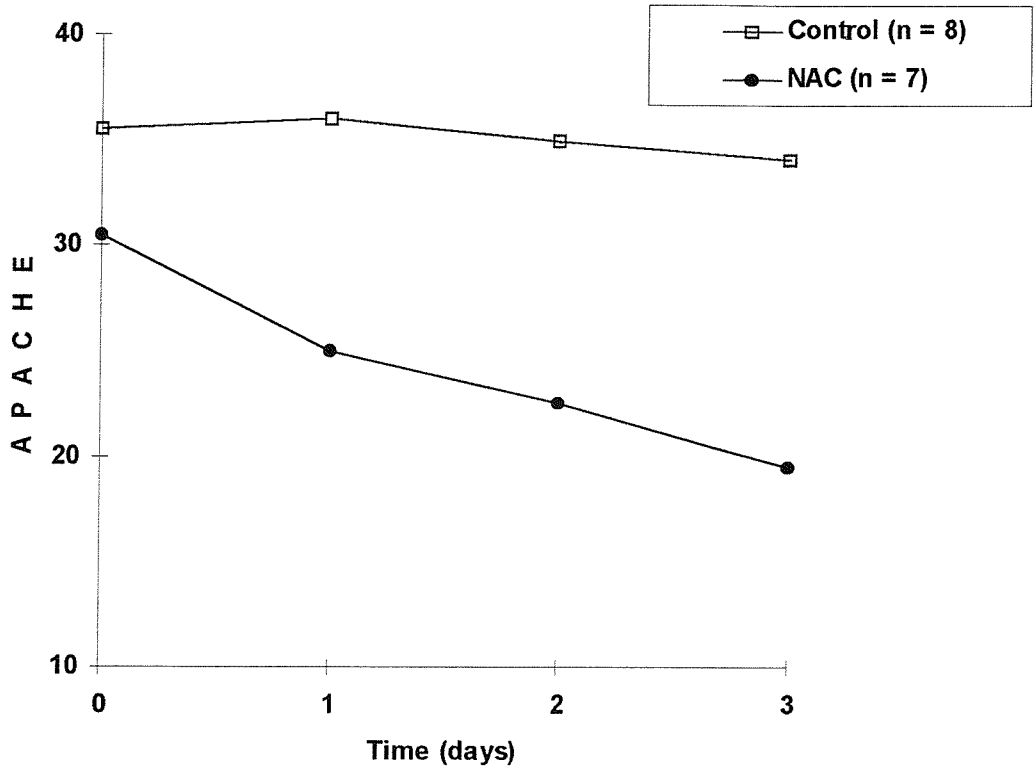


Figure 2.1. Median APACHE II scores in patients in the pilot randomised study of N-acetylcysteine (NAC) in multiple organ failure; all patients underwent intermittent haemodialysis to treat renal failure. From Braganza 1986b.

(Uden 1990). The successful combination included methionine (Figure 2.2) with selenium, vitamins C and E and β-carotene. These antioxidants were given because previous dietary studies had identified a lower intake of methionine, vitamin C and selenium in patients with idiopathic chronic pancreatitis compared to age and gender-matched controls (Rose 1986). A year later the Manchester group noted that pretreatment of rats with S-adenosylmethionine (SAMe) protected in an otherwise lethal model of allogenic pancreatitis (Scott 1992).

SAMe serves as the main methyl donor in cells, for membrane phospholipid methylation in signal transduction and its onward transmission towards GSH helps to replenish activated phosphate groups and sulphydryl (Finkelstein 1990). The vital importance of maintaining sufficient intracellular GSH in pancreatic homeostasis is observed experimentally. Inhibition of GSH synthesis is sufficient to trigger an attack of acute experimental pancreatitis (Lüthen 1994c), GSH depletion inhibits amylase release (Stenson 1983), as does folate deficiency (Balaghi 1995) and supplementation of GSH with the monoethyl ester ameliorates caerulein-induced pancreatitis (Neuschwander-Tetri 1992). Similarities between hepato-cellular and acinar cell function are observed for, SAMe ameliorates CCl_4 injury (Corrales 1992), pretreatment with SAMe in alcoholic liver disease protects GSH levels (Vendemiale 1989, Garcia-Ruiz 1995), GSH protects against acute liver injury induced by CCl4 in rats (Arosio 1997) and methionine limits alcohol-induced liver damage in rats (Parlesak 1998).

Thus we reasoned that the combination of SAMe and NAC (Figure 2.2) should bolster GSH levels and thus accelerate recovery from acute pancreatitis by overcoming pancreastasis in acinar cells, thereby removing the drive to inflammation from free radical inflammation products that are diverted into the interstitium along with normal pancreatic secretions (Braganza 1991). In addition to the potential effects on GSH, methionine and NAC help protect extra-cellular proteases against phagocyte-derived oxidants, which are implicated in the dissemination of tissue injury in acute pancreatitis (Read 1976) and NAC has been shown recently (after

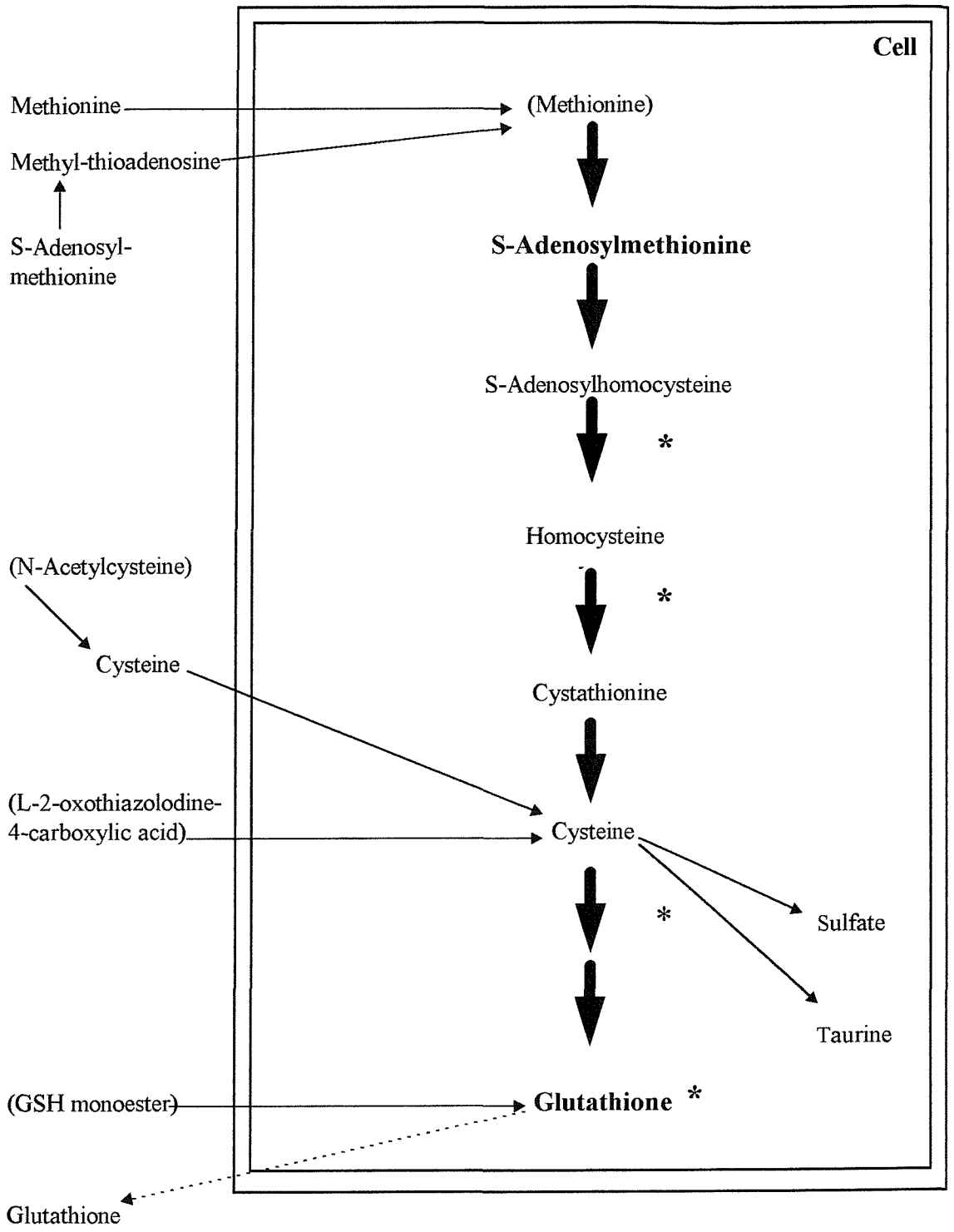


Figure 2.2. Schematic representation of the intracellular trans-sulphuration pathway of methionine metabolism, showing points that are vulnerable to oxidative stress (*) and compounds that have been used in human or experimental (bracketed) studies. GSH = glutathione in the reduced bioactive form.

completion of the studies described here) to decrease the severity of experimental pancreatitis in mice (Demols 2000).

We therefore undertook a randomised trial to test this deduction and its corollary, that treatment need not be protracted if it can be given with minimum delay, inferences that seemed justified by the earlier experiences and animal studies published up to that time.

2.2.0. Methods

2.2.1. Pre-study power calculation.

In designing this study the assumption was made, that active treatment would reduce mortality by fifty percent. This optimism seemed justified given the results of the pilot study (Figure 2.1). It was calculated that to achieve a power of 0.9 in the clinical study that 80 patients would need to be recruited and 40 entered into the two limbs (control and active).

2.2.2. Ethical approval.

Ethical permission was obtained from the ethical committees of Central Manchester, Trafford and Tameside Hospitals. Informed written consent was obtained from all patients entered into the study. Consecutive patients with a diagnosis of acute pancreatitis were considered for entry at each hospital.

2.2.3. Inclusion criteria

The diagnosis of acute pancreatitis was accepted if the serum amylase level exceeded 10000 U/L (Phadebas method, normal < 300 U/L) in a patient with acute abdominal pain and a compatible clinical picture.

2.2.4. Exclusion criteria

Patients were excluded if they were < 16 years of age, pregnant or already taking antioxidant supplements. A previous attack of acute pancreatitis or established chronic pancreatitis was also considered an exclusion criteria, as it is an observation that mortality is lower in such patients than in those having a first attack, Read

1975. There was no exclusion of elderly patients, unless they declined or were unable to give informed consent.

2.2.5. Study design

A consensus meeting produced useful guidelines for clinical trials, endorsing the use of the APACHE II system for gauging severity at admission (Bradley 1993). Patients were stratified into prognostically 'mild' or 'severe' groups as predicted at the time of admission on APACHE II scores. Randomisation then assigned the patient to either 'control' or 'active' arms by the use of pre-sealed numbered envelopes held at each recruitment centre.

The serum amylase level is known to be unreliable in assessing disease severity of acute pancreatitis and other combinations of biochemical markers are also unhelpful until 48 hours later. Contrast-enhanced computed-tomography has been recommended, but this facility was not available for regular use in all the Manchester hospitals at that time. We decided to use admission APACHE II scores regarding scores of < (less than) or > (equal to or greater than) 8 as predictive of a mild or severe outcome, respectively. This cut-off point was chosen because a published report in patients with intra-abdominal sepsis showed that those with admission score ranging from 6 to 10 had a high mortality than those with lower scores (Bohnen 1988). Others papers published around the same time suggested that inappropriate inflammatory responses may be the common denominator in multi-system organ failure (MSOF) whether from fulminant sepsis or pancreatitis (Braganza 1988d, Rinderknecht 1988).

2.2.6. Biochemical monitoring

At the start of this clinical trial, the method to measure blood GSH had not been developed within our laboratory, whereas the assay for SAMe which had been used in the placebo-controlled trial of oral antioxidant therapy in recurrent pancreatitis (Uden 1990a, 1992) was discarded because it necessitated the collection of bovine pineal glands, not a desirable task with the BSE epidemic at its height.

Where possible and with the consent of the patient, admission blood samples were taken for measurement of the micronutrient antioxidants; plasma vitamin C, serum selenium, α-tocopherol and β-carotene. Two marker of free radical activity were assessed, namely the ratio of 9, 11-linoleic acid to the parent compound 9, 12-linoleic acid, expressed as a molar ratio (%MR) and the percentage of plasma vitamin C in the inactive non-ascorbate form. The methodology of these assays are described in Appendix A. An incomplete set of samples from the patients entered into the study was accepted for practical reasons, particularly in the patients entered by the two non-Manchester Royal Infirmary sites and out of hours on all three sites.

2.2.7. Pharmacological intervention

N-acetylcysteine (NAC) was obtained from *Evans Medical Ltd.*, Surrey, England and administered by a constant intravenous infusion into a peripheral vein over the next 24 hours period, at a dose of 300 mg/kg in 500 ml of 5% dextrose.

S-adenosylmethionine (SAM) was a gift from *Bioresearch*, Milan, Italy. It was given concurrent to the NAC, but through a separate infusion site, in a dose of 43 mg/kg in 500 ml of 0.9% saline.

2.2.8. Primary outcome

The impact of treatment was gauged from the change in APACHE II scores at 48 hours compared with values recorded at admission.

2.2.9. Secondary outcomes

The secondary outcomes were:

i) the frequency with which patients developed complications associated with acute pancreatitis, namely organ failure be it single or multi-organ in distribution, pancreatic necrosis necessitating laparotomy, pseudocysts or pancreatic abscess and cholangitis.

ii) duration of hospital stay and

iii) mortality directly attributable to the acute pancreatitis.

2.2.10. Statistical analysis

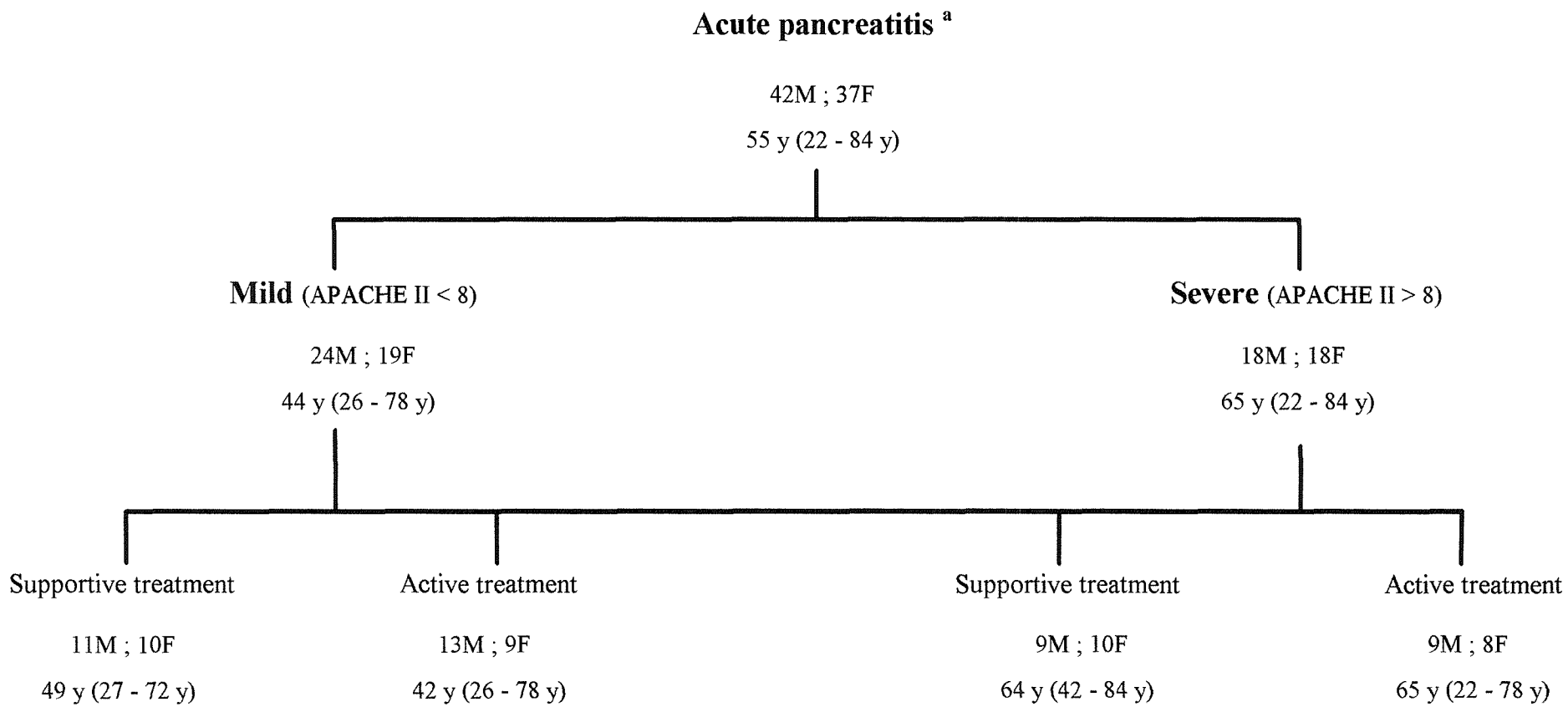
The χ^2 or Wilcoxon rank sum tests were used, as appropriate, to compare outcome measures in groups receiving supportive treatment or additional active treatment. The mortality and the change in APACHE II scores at 48 hours were further examined by logistic regression analysis. It was decided at the outset that the results would be re-analysed to take account of the time from first symptom to treatment. A time-lag of <15 hours was regarded as early, and > 15 hours as late, by analogy with paracetamol poisoning where it is recognised that treatment with NAC is more effective if administered early.

2.3.0 Results

2.3.1. Distribution of patients

The distribution of patients entered into the study is shown in Figure 2.3. The full data set of all patients entered into the trial appear in Table 2.1. These results are subsequently subdivided into; time at presentation (early / late) Table 2.2, therapy received (conventional [control] / active) Table 2.3 and disease prognosis at presentation (mild / severe) Table 2.4. Of the 80 patients entered into the trial, one was subsequently shown to have a perforated duodenal ulcer associated with gastric cancer diagnosed at laparotomy. This was done when the signs of peritonism increased a day after active treatment was given. This and the patient who self-discharged after 24 hours were the only patients in whom an ultrasound scan was not obtained.

The proportion of patients with mild to severe disease was 1.2 : 1. The following risk factors for acute pancreatitis were identified retrospectively: gallstones (n = 39), alcohol (n = 19), iatrogenic factors (n = 12) [drugs n = 5, cardiopulmonary bypass n = 4 and endoscopic retrograde cholangiopancreatography n = 1, endoscopic sphincterotomy for ascending cholangitis n = 2], and hypertriglyceridaemia (n = 2), Table 2.1. Two or more risk factors were identified in 9 patients, and in 14 patients the disease was regarded as idiopathic. Contrast enhanced computed tomography was done in 15 patients when deterioration suggested extensive pancreatic necrosis. In these circumstances ultra-sound-guided pancreatic aspirates were cultured to detect infected necrosis.



a = *A further patient with a perforated duodenal ulcer was excluded from the analysis*

Figure 2.3 Distribution of patients entered into trial.

initials	gender	age	time	therapy	severity	APOH	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
PR	f	54	e	a	m	2	3	hyperlip	n	85	1.84						
DP	f	26	l	a	m	2	0	gs	n	89	4.2						
NG	m	57	e	c	s	9	3	idio	n		4.51						
LM	f	43	e	c	m	5	3	gs	n		3.25						
CO	f	27	l	c	m	3	0	gs	n		3.79	6.6					
CS	f	61	l	a	s	12	4	alc	n	78	2.42						
JB	f	61	e	c	s	13	7	gs	n	106	4.16	5.6	3.80	0.68			
WS	m	50	e	c	m	2	2	alc	n		4.61	10.3	4.30	0.42			
AD	f	48	l	c	m	3	2	idio	n			6.5			170	7.53	12.33
AN	m	29	l	a	m	0	0	alc	n	71	3.43	2.1					
NK	m	64	l	c	s	11	6	gs	n								
SG	m	78	e	a	s	11	7	gs	y		2.7	0.3	0.01	0.03			
WR	m	65	l	a	s	8	13	gs	y								
KB	m	29	l	a	m	0	0	gs	n	63	3.85	1.8					
BS	m	38	l	a	m	6	3	alc	n	76	4.52	3.3	2.20	0.67	36	4.28	8.1
AS	m	32	e	a	m	2	0	gs	n		2.96	2.1	0.01	0.00			
AG	m	42	l	c	s	8	1	alc	n	28	3.87	1.2	0.01	0.01			
RK	f	62	l	c	s	15	11	ERCP	y		4.47	0.6	0.01	0.02			
FB	f	42	l	c	s	8	9	drugs/misc	n			5.9	0.30	0.05			
LW	m	59	e	c	m	7	7	idio	n	86	2.68	1.6	1.20	0.75			
PW	m	31	e	c	m	5	7	gs	n	100	2.05	5.3	2.80	0.53			
LH	f	71	e	c	s	8	6	gs	n	92	8.04				29		5.3
HG	f	49	e	c	m	4	2	gs	n		2.15	4.0					
HS	m	76	l	c	s	8	6	gs	n		6.4	8.7	0.01	0.00	59	4.29	9.3
AR	m	71	e	a	s	8	5	gs	n			1.9	3.00	1.58			
PR	f	42	l	a	m	1	0	gs	n	73	4.46	4.5	0.01	0.00	33	2.7	6.6
MH	f	69	l	c	m	7	6	gs	n	43	4.43				58	4.19	10.3
EG	f	76	l	a	s	8	8	gs	n	92	5.57	5.4	0.01	0.00	33	3.66	5.2

Table 2.1.

NAC / SAMe trial:
Individual patient data

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
CB	f	66	l	a	s	19	15	isch	n			3.2	0.01	0.00			
AW	m	49	e	a	s	26	22	isch	y	52	3.55	0.0	0.00		7	1.75	1.96
DD	f	58	l	c	s	8	5	gs	n		6.22	3.1	3.00	0.97			
CS	f	78	l	a	s	14	16	alc	n		1.49	1.6	0.01	0.01			
JG	m	68	l	c	m	7	6	gs		80	1.08	0.8	0.01	0.01	39	4.79	9.7
JS	m	38	e	c	m	2	1	alc	n	96	7.76	1.8	0.01	0.01	31	4.82	13.7
BB	f	72	l	c	m	5	5	idio	n	94	3.71	8.0			128	6.27	21.9
FB	f	34	l	a	m	2	5	idio	n	62	4.95	7.0	5.50	0.79	196	4.02	9.2
JO	m	65	l	a	m	7	7	gs/drugs	n	55	4.39	2.8	2.00	0.71	97	5.4	7.2
RB	m	63	e	c	m	3	3	gs	n	48	9.44	1.3	12.00	9.23	25	2.78	6.9
CV	f	69	e	a	s	10	x	gs	y	70	4.39	5.9			264	4.47	15.21
CM	m	40	l	a	m	4	2	alc	n								
PG	m	55	l	a	m	7	3	alc	n		3.58	4.0					
PJ	f	39	l	c	m	5	1	alc	n	63	3.74	2.9					
WL	m	59	e	c	s	12	11	idio	y								
KM	f	77	l	c	s	9	6	gs	n	34	3.43	5.4					
AR	f	84	l	c	s	8	10	idio	n	57	11.06	1.5					
EK	f	81	l	c	s	15	11	gs	n	60	5.09	2.7					
JS	f	51	l	c	m	4	3	gs	n	68	2.62	4.8					
IS	f	33	l	c	m	0	0	gs	n	79	1.93	3.7					
ST	m	32	l	a	m	0	0	alc	n	36	2.59	1.8	0.01	0.01	4	3.17	5.5
JT	m	50	l	c	s	9	9	idio	n	90	4.09	8.9	6.00	0.67	115	2.89	7.6
CM	m	38	e	c	m	1	x	alc	n		4.32	1.0			29	3.79	9.13
HB	m	77	l	a	m	6	6	Ca panc	y		1.62	7.4	2.90	0.39			
RE	m	46	l	c	m	5	2	gs	n								
TC	f	28	e	a	m	0	0	gs/drugs	n		2.79	3.6	0.01	0.00	15	3.19	8.94
NK	f	65	l	a	s	14	14	ERCP	n	64	4.15	4.1	3.40	0.83	26	3.78	6
JM	m	35	l	a	s	10	8	alc	n	59	1.85	2.0	0.01	0.01			

Table 2.1. (continued)

NAC / SAMe trial:
Individual patient data

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
CO	m	59	l	c	s	18	19	isch	n		3.46	4.6	5.10	1.11			
KW	m	41	l	a	m	7	4	alc	n	87	5.99	1.1	1.10	1.00	35	4.98	9.86
JF	m	49	l	a	m	6	2	alc	n	64	2.25	1.6	0.01	0.01	42	4.55	8.43
JM	f	75	l	c	s	10	8	idio	n	69	4.32	2.0					
EO	m	39	l	c	m	3	8	hyperlip	n	70	1.22	1.9	0.50	0.26	16	8.94	17.33
GM	f	36	l	a	m	3	0	alc	n	81	3.58	1.0					
KS	m	44	l	a	m	0	0	steroids	n	51	1.88	1.1			5	4.6	8.87
HD	m	66	e	a	s	10	6	gs	n	69	3.47	1.1					
FH	m	68	e	c	m	7	5	gs	n	67	2.4	4.4	3.20	0.73			
HS	f	51	l	a	m	4	3	idio	n	94	2.32	9.8	7.10	0.72			
BS	m	64	l	a	s	13	6	gs	y	35		2.3			8	7.09	5.5
DR	f	22	l	a	s	11	8	gs/drug/ob	y	27	2.3	2.5	2.50	1.00	4	6.76	2.9
MA	m	38	l	a	s	18	19	ischamia	y	64	2.05	6.6					
MO	f	54	l	a	m	2	2	drugs	n	77	3.51	7.8					
GF	m	43	l	a	m	0	2	idio	n	81	1.58	1.3					
JH	f	62	e	c	m	4	7	gs	n		5.01	6.9					
(GS)	m	78	l	c	s	17	NA	gastric Ca	y	NA	NA	NA			NA	NA	NA
CF	m	29	l	a	s	11	12	alc	n	52	2.46	7.3	3.40	0.47	3	4.8	4.95
MH	f	79	l	c	s	8	4	idio	n	70	4.89	1.9					
NB	f	53	e	a	s	16	7	gs	n	33	3.24	6.2					
FH	m	78	l	a	m	7	6	ERCP	n	60	5.58	2.3					
NK	m	70	l	c	s	10	14	idio	y	68	3.6	1.6			148	4.21	8.8
RM	m	55	l	c	m	6	2	alc	n	50	5.57	0.5					
mean		53.9				7.3	5.7			67.7	3.8	3.7	2.04	0.66	59.1	4.6	8.8
median		54.0				7.0	5.0			68.5	3.6	2.9	1.10	0.40	33.0	4.3	8.6

Table 2.1. (continued)

NAC / SAMe trial:
Individual patient data

Key to Table 2.1.

AP0H	APACHE II score at time zero
AP48H	APACHE II score at 48 hours
Sel	Serum selenium µg/l
%MR	Percentage molar ratio 9,11 / 9,12 Linoleic acid
Vit C	Plasma Vitamin C mg/l
NA	Not available
AA	Plasma ascorbic acid mg/l
a/vc	Plasma ascorbic acid / vitamin C ratio
β-car	Serum β-carotene µm/l
vit E/chol	Ratio serum β-carotene to cholesterol
vit E	Serum Vitamin E mg/l

Table 2.1. (continued)

NAC / SAMe trial:
Individual patient data

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
PR	f	54	e	a	m	2	3	hyperlip	n	85	1.84						
NG	m	57	e	c	s	9	3	idio	n		4.51						
LM	f	43	e	c	m	5	3	gs	n		3.25						
JB	f	61	e	c	s	13	7	gs	n	106	4.16	5.6	3.80	0.68			
WS	m	50	e	c	m	2	2	alc	n		4.61	10.3	4.30	0.42			
SG	m	78	e	a	s	11	7	gs	y		2.7	0.3	0.01	0.03			
AS	m	32	e	a	m	2	0	gs	n		2.96	2.1	0.01	0.00			
LW	m	59	e	c	m	7	7	idio	n	86	2.68	1.6	1.20	0.75			
PW	m	31	e	c	m	5	7	gs	n	100	2.05	5.3	2.80	0.53			
LH	f	71	e	c	s	8	6	gs	n	92	8.04	0	0.00		29		5.3
HG	f	49	e	c	m	4	2	gs	n		2.15	4					
AR	m	71	e	a	s	8	5	gs	n			1.9	3.00	1.58			
AW	m	49	e	a	s	26	22	isch	y	52	3.55	0	0.00		7	1.75	1.96
JS	m	38	e	c	m	2	1	alc	n	96	7.76	1.8	0.01	0.01	31	4.82	13.7
RB	m	63	e	c	m	3	3	gs	n	48	9.44	1.3	1.20	0.92	25	2.78	6.9
CV	f	69	e	a	s	10		gs	y	70	4.39	5.9			264	4.47	15.21
WL	m	59	e	c	s	12	11	idio	y								
CM	m	38	e	c	m	1		alc	n		4.32	1			29	3.79	9.13
TC	f	28	e	a	m	0	0	gs/drugs	n		2.79	3.6	0.01	0.00	15	3.19	8.94
HD	m	66	e	a	s	10	6	gs	n	69	3.47	1.1					
FH	m	68	e	c	m	7	5	gs	n	67	2.4	4.4	3.20	0.73			
JH	f	62	e	c	m	4	7	gs	n		5.01	6.9					
NB	f	53	e	a	s	16	7	gs	n	33	3.24	6.2					
means		54.30				7.26	5.43			75.33	4.06	3.33	1.50	0.51	57.14	3.47	8.73
median		57.0				7.0	5.0			77.5	3.5	2.1	1.20	0.53	29.0	3.5	8.9

Table 2.2.i.

NAC / SAMe Trial
Early presentation

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	b-car	vit E/chol	vit E
DP	f	26	1	a	m	2	0		n	89	4.2						
CO	f	27	1	c	m	3	0		n		3.79	6.6					
CS	f	61	1	a	s	12	4	alc	n	78	2.42						
AD	f	48	1	c	m	3	2	idio	n			6.5			170	7.53	12.33
AN	m	29	1	a	m	0	0	alc	n	71	3.43	2.1					
NK	m	61	1	c	s	11	6		n								
WR	m	65	1	a	s	8	13		y								
KB	m	29	1	a	m	0	0		n	63	3.85	1.8					
BS	m	38	1	a	m	6	3	alc	n	76	4.52	3.3	2.2	0.6667	36	4.28	8.1
AG	m	42	1	c	s	8	1	alc	n	28	3.87	1.2	<0.5				
RK	f	62	1	c	s	15	11		y		4.47	0.6	<0.5				
FB	f	42	1	c	s	8	9		n			5.9	0.3	0.0508			
HS	m	76	1	c	s	8	6		n		6.4	8.7	<0.5		59	4.29	9.3
PR	f	42	1	a	m	1	0		n	73	4.46	4.5	<0.5		33	2.7	6.6
MH	f	69	1	c	m	7	6		n	43	4.43				58	4.19	10.3
EG	f	76	1	a	s	8	8		n	92	5.57	5.4	<0.5		33	3.66	5.2
CB	f	66	1	a	s	19	15	isch	n			3.2	<0.5				
DD	f	58	1	c	s	8	5		n		6.22	3.1	3	0.9677			
CS	f	78	1	a	s	14	16	alc	n		1.49	1.6	<0.5				
JG	m	68	1	c	m	7	6			80	1.08	0.8	<0.5		39	4.79	9.7
BB	f	72	1	c	m	5	5	idio	n	94	3.71	8			128	6.27	21.9
FB	f	34	1	a	m	2	5	idio	n	62	4.95	7	5.5	0.7857	196	4.02	9.2
JO	m	65	1	a	m	7	7		n	55	4.39	2.8	2	0.7143	97	5.4	7.2
CM	m	40	1	a	m	4	2	alc	n								
PG	m	55	1	a	m	7	3	alc	n		3.58	4					
PJ	f	39	1	c	m	5	1	alc	n	63	3.74	2.9					
KM	f	77	1	c	s	9	6		n	34	3.43	5.4					
AR	f	84	1	c	s	8	10	idio	n	57	11.06	1.5					
EK	f	81	1	c	s	15	11		n	60	5.09	2.7					

NAC / SAMe Trial

initials	gender	age	time	therapy	severity	AP0H	AP48H	aeti	died	sel	%MR	Vit C	AA	a/vc	b-car	vit E/chol	vit E
JS	f	51	1	c	m	4	3		n	68	2.62	4.8					
IS	f	33	1	c	m	0	0		n	79	1.93	3.7					
ST	m	32	1	a	m	0	0	alc	n	36	2.59	1.8	<0.5		4	3.17	5.5
JT	m	50	1	c	s	9	9		n	90	4.09	8.9	6	0.6742	115	2.89	7.6
HB	m	77	1	a	m	6	6	Ca panc	y		1.62	7.4	2.9	0.3919			
RE	m	46	1	c	m	5	2		n								
NK	f	65	1	a	s	14	14		n	64	4.15	4.1	3.4	0.8293	26	3.78	6
JM	m	35	1	a	s	10	8	alc	n	59	1.85	2	<0.5				
CO	m	59	1	c	s	18	19	isch	n		3.46	4.6	5.1	1.1087			
KW	m	41	1	a	m	7	4	alc	n	87	5.99	1.1	1.1	1	35	4.98	9.86
JF	m	49	1	a	m	6	2	alc	n	64	2.25	1.6	0.05	0.0313	42	4.55	8.43
JM	f	75	1	c	s	10	8	idio	n	69	4.32	2					
EO	m	39	1	c	m	3	8	hyperlip	n	70	1.22	1.9	0.5	0.2632	16	8.94	17.33
GM	f	36	1	a	m	3	0	alc	n	81	3.58	1					
KS	m	44	1	a	m	0	0	steroids	n	51	1.88	1.1			5	4.6	8.87
HS	f	51	1	a	m	4	3		n	94	2.32	9.8	7.1	0.7245			
BS	m	64	1	a	s	13	6		y	35		2.3			8	7.09	5.5
DR	f	22	1	a	s	11	8		y	27	2.3	2.5	2.5		4	6.76	2.9
MA	m	38	1	a	s	18	19		y	64	2.05	6.6					
MO	f	54	1	a	m	2	2		n	77	3.51	7.8					
GF	m	43	1	a	m	0	2	idio	n	81	1.58	1.3					
GS	m	78	1	c	s	17			y	67	4.53	1.8			2	3.58	5.4
CF	m	29	1	a	s	11	12	alc	n	52	2.46	7.3	3.4	0.4658	3	4.8	4.95
MH	f	79	1	c	s	8	4	idio	n	70	4.89	1.9					
FH	m	78	1	a	m	7	6		n	60	5.58	2.3					
NK	m	70	1	c	s	10	14	idio	y	68	3.6	1.6			148	4.21	8.8
RM	m	55	1	c	m	6	2	alc	n	50	5.57	0.5					
mean		53.63				7.36	5.85			65.39	3.75	3.70	3.00	0.8117	57.14	4.84	8.68
median		52.50				7.00	5.00			67.00	3.73	2.80	2.90	1.0357	35.50	4.42	8.27

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
NG	m	57	e	c	s	9	3	idio	n		4.51						
LM	f	43	e	c	m	5	3	gs	n		3.25						
CO	f	27	l	c	m	3	0	gs	n		3.79	6.6					
JB	f	61	e	c	s	13	7	gs	n	106	4.16	5.6	3.80	0.68			
WS	m	50	e	c	m	2	2	alc	n		4.61	10.3	4.30	0.42			
AD	f	48	l	c	m	3	2	idio	n			6.5			170	7.53	12.33
NK	m	61	l	c	s	11	6	gs	n								
AG	m	42	l	c	s	8	1	alc	n	28	3.87	1.2	0.01	0.01			
RK	f	62	l	c	s	15	11	ERCP	y		4.47	0.6	0.01	0.02			
FB	f	42	l	c	s	8	9	drugs/misc	n			5.9	0.30	0.05			
LW	m	59	e	c	m	7	7	idio	n	86	2.68	1.6	1.20	0.75			
PW	m	31	e	c	m	5	7	gs	n	100	2.05	5.3	2.80	0.53			
LH	f	71	e	c	s	8	6	gs	n	92	8.04	0	0.00		29		5.3
HG	f	49	e	c	m	4	2	gs	n		2.15	4					
HS	m	76	l	c	s	8	6	gs	n		6.4	8.7	0.01	0.00	59	4.29	9.3
MH	f	69	l	c	m	7	6	gs	n	43	4.43				58	4.19	10.3
DD	f	58	l	c	s	8	5	gs	n		6.22	3.1	3.00	0.97			
JG	m	68	l	c	m	7	6	gs		80	1.08	0.8	0.01	0.01	39	4.79	9.7
JS	m	38	e	c	m	2	1	alc	n	96	7.76	1.8	0.01	0.01	31	4.82	13.7
BB	f	72	l	c	m	5	5	idio	n	94	3.71	8			128	6.27	21.9
RB	m	63	e	c	m	3	3	gs	n	48	9.44	1.3	1.20	0.92	25	2.78	6.9
PJ	f	39	l	c	m	5	1	alc	n	63	3.74	2.9					

NAC / SAMe Trial

Control therapy

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	Sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
WL	m	59	e	c	s	12	11	idio	y								
KM	f	77	l	c	s	9	6	gs	n	34	3.43	5.4					
AR	f	84	l	c	s	8	10	idio	n	57	11.06	1.5					
EK	f	81	I	c	s	15	11	gs	n	60	5.09	2.7					
JS	f	51	l	c	m	4	3	gs	n	68	2.62	4.8					
IS	f	33	l	c	m	0	0	gs	n	79	1.93	3.7					
JT	m	50	l	c	s	9	9	idio	n	90	4.09	8.9	6.00	0.67	115	2.89	7.6
CM	m	38	e	c	m	1		alc	n		4.32	1			29	3.79	9.13
RE	m	46	l	c	m	5	2	gs	n								
CO	m	59	l	c	s	18	19	isch	n		3.46	4.6	5.10	1.11			
JM	f	75	l	c	s	10	8	idio	n	69	4.32	2					
EO	m	39	l	c	m	3	8	hyperlip	n	70	1.22	1.9	0.50	0.26	16	8.94	17.33
FH	m	68	e	c	m	7	5	gs	n	67	2.4	4.4	3.20	0.73			
JH	f	62	e	c	m	4	7	gs	n		5.01	6.9					
GS	m	78	l	c	s	17		gastric Ca	y	67	4.53	1.8			2	3.58	5.4
MH	f	79	l	c	s	8	4	idio	n	70	4.89	1.9					
NK	m	70	l	c	s	10	14	idio	y	68	3.6	1.6			148	4.21	8.8
RM	m	55	l	c	m	6	2	alc	n	50	5.57	0.5					
means		57.25				7.30	5.74			70.21	4.40	3.87	1.97	0.45	0.45	65.31	4.84
median		59.0				7.0	6.0			68.5	4.2	3.1	1.20	0.47	0.5	39.0	4.3

NAC / SAMe Trial
Control therapy

Table 2.3.i. (continued)

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
PR	f	54	e	a	m	2	3	hyperlip	n	85	1.84						
DP	f	26	l	a	m	2	0	gs	n	89	4.2						
CS	f	61	l	a	s	12	4	alc	n	78	2.42						
AN	m	29	l	a	m	0	0	alc	n	71	3.43	2.1					
SG	m	78	e	a	s	11	7	gs	y		2.7	0.3	0.01	0.03			
WR	m	65	l	a	s	8	13	gs	y								
KB	m	29	l	a	m	0	0	gs	n	63	3.85	1.8					
BS	m	38	l	a	m	6	3	alc	n	76	4.52	3.3	2.20	0.67	36	4.28	8.1
AS	m	32	e	a	m	2	0	gs	n		2.96	2.1	0.01	0.00			
AR	m	71	e	a	s	8	5	gs	n			1.9	3.00	1.58			
PR	f	42	l	a	m	1	0	gs	n	73	4.46	4.5	0.01	0.00	33	2.7	6.6
EG	f	76	l	a	s	8	8	gs	n	92	5.57	5.4	0.01	0.00	33	3.66	5.2
CB	f	66	l	a	s	19	15	isch	n			3.2	0.01	0.00			
AW	m	49	e	a	s	26	22	isch	y	52	3.55				7	1.75	1.96
CS	f	78	l	a	s	14	16	alc	n		1.49	1.6	0.01	0.01			
FB	f	34	l	a	m	2	5	idio	n	62	4.95	7.0	5.50	0.79	196	4.02	9.2
JO	m	65	l	a	m	7	7	gs/drugs	n	55	4.39	2.8	2.00	0.71	97	5.4	7.2
CV	f	69	e	a	s	10		gs	y	70	4.39	5.9			264	4.47	15.21
CM	m	40	l	a	m	4	2	alc	n								
PG	m	55	l	a	m	7	3	alc	n		3.58	4.0					
ST	m	32	l	a	m	0	0	alc	n	36	2.59	1.8	0.01	0.01	4	3.17	5.5

NAC / SAMe Trial

Active therapy

Table 2.3 ::

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
HB	m	77	l	a	m	6	6	Ca panc	y		1.62	7.4	2.90	0.39			
TC	f	28	e	a	m	0	0	gs/drugs	n		2.79	3.6	0.01	0.00	15	3.19	8.94
NK	f	65	l	a	s	14	14	ERCP	n	64	4.15	4.1	3.40	0.83	26	3.78	6
JM	m	35	l	a	s	10	8	alc	n	59	1.85	2.0	0.01	0.01			
KW	m	41	l	a	m	7	4	alc	n	87	5.99	1.1	1.10	1.00	35	4.98	9.86
JF	m	49	l	a	m	6	2	alc	n	64	2.25	1.6	0.01	0.01	42	4.55	8.43
GM	f	36	l	a	m	3	0	alc	n	81	3.58	1.0					
KS	m	44	l	a	m	0	0	steroids	n	51	1.88	1.1			5	4.6	8.87
HD	m	66	e	a	s	10	6	gs	n	69	3.47	1.1					
HS	f	51	l	a	m	4	3	idio	n	94	2.32	9.8	7.10	0.72			
BS	m	64	l	a	s	13	6	gs	y	35		2.3			8	7.09	5.5
DR	f	22	l	a	s	11	8	gs/drug/ob	y	27	2.3	2.5	2.50	1.00	4	6.76	2.9
MA	m	38	l	a	s	18	19	ischamia	y	64	2.05	6.6					
MO	f	54	l	a	m	2	2	drugs	n	77	3.51	7.8					
GF	m	43	l	a	m	0	2	idio	n	81	1.58	1.3					
CF	m	29	l	a	s	11	12	alc	n	52	2.46	7.3	3.40	0.47	3	4.8	4.95
NB	f	53	e	a	s	16	7	gs	n	33	3.24	6.2					
FH	m	78	l	a	m	7	6	ERCP	n	60	5.58	2.3					
mean		50.31				7.36	5.74			65.52	3.28	3.5	1.66	0.41	50.50	4.33	7.15
median		49.0				7.0	4.5			64.0	3.3	2.5	0.56	0.21	29.5	4.4	6.9

NAC / SAMe Trial
Active therapy

Table 2.3 ii (continued)

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
PR	f	54	e	a	m	2	3	hyperlip	n	85	1.84						
DP	f	26	l	a	m	2	0	gs	n	89	4.2						
LM	f	43	e	c	m	5	3	gs	n		3.25						
CO	f	27	l	c	m	3	0	gs	n		3.79	6.6					
WS	m	50	e	c	m	2	2	alc	n		4.61	10.3	4.3	0.42			
AD	f	48	l	c	m	3	2	idio	n			6.5			170	7.53	12.33
AN	m	29	l	a	m	0	0	alc	n	71	3.43	2.1					
KB	m	29	l	a	m	0	0	gs	n	63	3.85	1.8					
BS	m	38	l	a	m	6	3	alc	n	76	4.52	3.3	2.2	0.67	36	4.28	8.1
AS	m	32	e	a	m	2	0	gs	n		2.96	2.1	0.01	0.00			
LW	m	59	e	c	m	7	7	idio	n	86	2.68	1.6	1.2	0.75			
PW	m	31	e	c	m	5	7	gs	n	100	2.05	5.3	2.8	0.53			
HG	f	49	e	c	m	4	2	gs	n		2.15	4					
PR	f	42	l	a	m	1	0	gs	n	73	4.46	4.5	0.01	0.00	33	2.7	6.6
MH	f	69	l	c	m	7	6	gs	n	43	4.43				58	4.19	10.3
JG	m	68	l	c	m	7	6	gs		80	1.08	0.8	0.01	0.01	39	4.79	9.7
JS	m	38	e	c	m	2	1	alc	n	96	7.76	1.8	0.01	0.01	31	4.82	13.7
BB	f	72	l	c	m	5	5	idio	n	94	3.71	8			128	6.27	21.9
FB	f	34	l	a	m	2	5	idio	n	62	4.95	7	5.5	0.79	196	4.02	9.2
JO	m	65	l	a	m	7	7	gs/drugs	n	55	4.39	2.8	2	0.71	97	5.4	7.2
RB	m	63	e	c	m	3	3	gs	n	48	9.44	1.3	12	9.23	25	2.78	6.9
CM	m	40	l	a	m	4	2	alc	n								
PG	m	55	l	a	m	7	3	alc	n		3.58	4					

Table 2.4.i. **NAC / SAMe Trial**
Mild AP

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
PJ	f	39	1	c	m	5	1	alc	n	63	3.74	2.9					
JS	f	51	1	c	m	4	3	gs	n	68	2.62	4.8					
IS	f	33	1	c	m	0	0	gs	n	79	1.93	3.7					
ST	m	32	1	a	m	0	0	alc	n	36	2.59	1.8	0.01		4	3.17	5.5
CM	m	38	e	c	m	1		alc	n		4.32	1			29	3.79	9.13
HB	m	77	1	a	m	6	6	Ca panc	y		1.62	7.4	2.9	0.39			
RE	m	46	1	c	m	5	2	gs	n								
TC	f	28	e	a	m	0	0	gs/drugs	n		2.79	3.6	0.01		15	3.19	8.94
KW	m	41	1	a	m	7	4	alc	n	87	5.99	1.1	1.1	1.00	35	4.98	9.86
JF	m	49	1	a	m	6	2	alc	n	64	2.25	1.6	0.01		42	4.55	8.43
EO	m	39	1	c	m	3	8	hyperlip	n	70	1.22	1.9	0.5	0.26	16	8.94	17.33
GM	f	36	1	a	m	3	0	alc	n	81	3.58	1					
KS	m	44	1	a	m	0	0	steroids	n	51	1.88	1.1			5	4.6	8.87
FH	m	68	e	c	m	7	5	gs	n	67	2.4	4.4	3.2	0.73			
HS	f	51	1	a	m	4	3	idio	n	94	2.32	9.8	7.1	0.72			
MO	f	54	1	a	m	2	2	drugs	n	77	3.51	7.8					
GF	m	43	1	a	m	0	2	idio	n	81	1.58	1.3					
JH	f	62	e	c	m	4	7	gs	n		5.01	6.9					
FH	m	78	1	a	m	7	6	ERCP	n	60	5.58	2.3					
RM	m	55	1	c	m	6	2	alc	n	50	5.57	0.5					
mean		47.09				3.63	2.86			71.63	3.59	3.75	2.36	1.35	56.41	4.71	10.23
median		44				4	2			72	3.545	2.9	1.2	0.72	35	4.55	9.13

NAC / SAMe Trial
Mild AP

Table 2.4.i. (continued)

initials	gender	age	time	therapy	severity	APOH	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
NG	m	57	e	c	s	9	3	idio	n		4.51						
CS	f	61	l	a	s	12	4	alc	n	78	2.42						
JB	f	61	e	c	s	13	7	gs	n	106	4.16	5.6	3.80	0.68			
NK	m	61	l	c	s	11	6	gs	n								
SG	m	78	e	a	s	11	7	gs	y		2.7	0.3	0.01				
WR	m	65	l	a	s	8	13	gs	y								
AG	m	42	l	c	s	8	1	alc	n	28	3.87	1.2	0.01				
RK	f	62	l	c	s	15	11	ERCP	y		4.47	0.6	0.01				
FB	f	42	l	c	s	8	9	drug/misc	n			5.9	0.30	0.05			
LH	f	71	e	c	s	8	6	gs	n	92	8.04				29		5.3
HS	m	76	l	c	s	8	6	gs	n		6.4	8.7	0.01		59	4.29	9.3
AR	m	71	e	a	s	8	5	gs	n			1.9	3.00	1.58			
EG	f	76	l	a	s	8	8	gs	n	92	5.57	5.4	0.01		33	3.66	5.2
CB	f	66	l	a	s	19	15	isch	n			3.2	0.01				
AW	m	49	e	a	s	26	22	isch	y	52	3.55				7	1.75	1.96
DD	f	58	l	c	s	8	5	gs	n		6.22	3.1	3.00	0.97			
CS	f	78	l	a	s	14	16	alc	n		1.49	1.6	0.01				
CV	f	69	e	a	s	10		gs	y	70	4.39	5.9			264	4.47	15.21
WL	m	59	e	c	s	12	11	idio	y								

Table 2.4.ii.

NAC / SAMe Trial
Severe AP

initials	gender	age	time	therapy	severity	AP0H	AP48H	aetiology	died	sel	%MR	Vit C	AA	a/vc	β-car	vit E/chol	vit E
KM	f	77	1	c	s	9	6	gs	n	34	3.43	5.4					
AR	f	84	1	c	s	8	10	idio	n	57	11.06	1.5					
EK	f	81	1	c	s	15	11	gs	n	60	5.09	2.7					
JT	m	50	1	c	s	9	9	idio	n	90	4.09	8.9	6.00	0.67	115	2.89	7.6
NK	f	65	1	a	s	14	14	ERCP	n	64	4.15	4.1	3.40	0.83	26	3.78	6
JM	m	35	1	a	s	10	8	alc	n	59	1.85	2	0.01				
CO	m	59	1	c	s	18	19	isch	n		3.46	4.6	5.10	1.11			
JM	f	75	1	c	s	10	8	idio	n	69	4.32	2					
HD	m	66	e	a	s	10	6	gs	n	69	3.47	1.1					
BS	m	64	1	a	s	13	6	gs	y	35		2.3			8	7.09	5.5
DR	f	22	1	a	s	11	8	gs/drug/ob	y	27	2.3	2.5	2.50	1.00	4	6.76	2.9
MA	m	38	1	a	s	18	19	ischamia	y	64	2.05	6.6					
GS	m	78	1	c	s	17		gastric Ca	y	67	4.53	1.8			2	3.58	5.4
CF	m	29	1	a	s	11	12	alc	n	52	2.46	7.3	3.40	0.47	3	4.8	4.95
MH	f	79	1	c	s	8	4	idio	n	70	4.89	1.9					
NB	f	53	e	a	s	16	7	gs	n	33	3.24	6.2					
NK	m	70	1	c	s	10	14	idio	y	68	3.6	1.6			148	4.21	8.8
mean		61.86				11.75	9.29			62.43	4.20	3.65	1.80	0.82	58.17	4.30	6.51
median		64.5				10.5	8			64	4.09	2.7	0.30	0.83	27.5	4.21	5.45

Table 2.4.ii. (continued)

NAC / SAMe Trial
Severe AP

2.3.2. Trial outcomes

Treatment with SAMe and NAC did not influence recovery, whether the preset criteria were considered individually (Table 2.5) or by logistic regression analysis. Although there were no deaths among patients with admission APACHE I scores < 8, 26 % of them developed a local complication. Of these, 5 had extensive pancreatic necrosis, 50% as shown by computed tomography, and went on to develop the adult respiratory distress syndrome (ARDS); laparostomy with repeat surgical repacking under general anaesthesia was undertaken in these patients, with respiratory support as needed.

The patent who required 129 days in hospital (Table 2.6) had gallstone-related disease, developed a pancreatic abscess, went on to laparostomy and later developed an enter-cutaneous fistula and septicaemia from methicillin-resistant *Staphylococcus aureus*, although his admission APACHE II score was zero.

The median length of stay in patients with admission APACHE II scores >7 was roughly twice that in patients who were regarded as having mild disease at the outset, Table 2.5.

Eight patents with an admission APACHE II score of 8 or more died, 4 in the subgroup receiving conventional supportive therapy (controls) and 4 in the subgroup receiving additional active treatment.

2.3.3. Influence of time-lag to treatment

Analysis of the time period between the onset of the initial symptoms and treatment was begun (Table 2.6), showed that only 29% of patients presented within 15 hours of the onset of pain. The shortest interval of 4 hours was in a patient in whom acute pancreatitis was precipitated by endoscopic sphincterotomy.

Active treatment did not result in a significant improvement in outcome, whether given within or after 15 hours from the first symptom. However, it is noticeable

that although the median APACHE II score at entry in the subgroup given active treatment within 15 hours was twice that in the corresponding subgroup on supportive treatment (10 and 5 days respectively), the time to discharge from hospital was similar in these subgroups (7 and 8 days respectively).

Among the deaths, 1 occurred in a patient who presented within 15 hours and was on supportive treatment and the other 7 deaths occurred in patients who arrive later.

2.3.4. Cause of death

When information on mortality was sought from hospital records department of the 3 hospitals, 12 deaths were notified. However, in only 8 cases could the death be reasonably be ascribed to the pancreatitis. The causes of death appear in Table 2.7.

Group	Treatment	No. of pts	Entry APACHE II[a]	Reduced APACHE II score at 48h (%patients)[b]	Days to discharge	Complications (no of patients)[c]	Deaths attributed to pancreatitis
Mild (APACHE <8) [n = 43]	Supportive	21	4 (0 - 7)	75	8 (2 - 92)	Pseudocysts (2) Abscess (1) Cholangitis (1) Pancreatic necrosis /ARDS (1)	0
	Active	22	2 (0 - 7)	73	8 (6 - 129)	Pseudocysts (1) Abscess (1) Pseudocysts/pancreatic necrosis (1) Abscess/pancreatic necrosis (1) Pancreatic necrosis (1) Pancreatic necrosis /ARDS (1)	0
Severe (APACHE >8) [n = 36]	Supportive	19	9 (8 - 18)	72	15 (7 - 23)	Cholangitis (1) MSOF (1)	4
	Active	17	11 (8 - 26)	69	15 (6 - 60)	Cholangitis (1) Abscess (1) Pancreatic necrosis /ARDS (1) ARDS (1)	4

a Results presented as mean (range)

b Excluding patients with an APACHE II score that was unable to improve, i.e. zero or entirely attributed to age, the 2 deaths within 48 hours and 1 patient who discharged himself

c Excluding deaths. Pancreatic necrosis, > 50% of parenchyma on CT, always resulted in necrosectomy and laparostomy. Peritoneal dialysis, haemodialysis or haemofiltration were implemented as needed in 1 pts with multisystem organ failure (MSOF) and in 2 pts with adult respiratory distress syndrome (ARDS).

Table 2.5. **NAC / SAMe Trial outcome**

Group	Treatment and time-lag (h) [a]	No. of pts	Entry APACHE II [a]	Reduced APACHE II score at 48h (%patients) [b]	Days to discharge [a]	Deaths attributed to pancreatitis
Early (< 15 h) [n = 23]	Supportive 9 (4 - 14)	14	5 (1 - 13)	73	8 (2 - 92)	1
	Active 11 (9 - 14)	9	10 (0 - 26)	86	7 (6 - 60)	0
Late (> 15 h) [n = 56]	Supportive 48 (18 - 200)	26	8 (0 - 18)	74	8 (6 - 79)	3
	Active 72 (18 - >240)	30	7 (0 - 19)	67	13 (6 - 129)	4

a Results presented as median (range) for interval from first symptom (hours) and entry APACHE II scores.
b Excluding patients with an APACHE II score that was unable to improve, i.e. zero or entirely attributed to age, the 2 deaths within 48 hours and 1 patient who discharged himself.

Table 2.6. **Influence of time-lag to treatment in NAC / SAMe Trial**

Index No.	Treatment group	Cause	Time from admission (days)	Clinical complication
Details of deaths attributable to acute pancreatitis				
1	Supportive treatment severe pancreatitis early presentation	MSOF	28	Laparostomy, drainage, pseudocyst, necrosectomy and multiple debridements / lavages, haemodialysis.
2	Supportive treatment severe pancreatitis late presentation	MSOF	2	Laparotomy for haematemesis, gastric neoplasm found at site of previous gastrojejunostomy, occluding duodenum in a blind loop.
3	Supportive treatment severe pancreatitis late presentation	MSOF / DIC	3	Laparostomy and necrosectomy.
4	Supportive treatment severe pancreatitis late presentation	ARDS / CVA	51	Precipitated by ERCP and sphincterotomy; cholecystectomy and choledochoduodenostomy but bile leak, second laparotomy, ARDS.
5	Active treatment severe pancreatitis late presentation	GI bleed, shock, cardiac failure	3	Haemorrhagic pancreatic pseudocyst and acute GI bleed.
6	Active treatment severe pancreatitis late presentation	MSOF, sepsis	15	Shock 2 days post CABG and LV aneurysectomy, peritoneal dialysis, Gram-negative sepsis.
7	Active treatment severe pancreatitis late presentation	MSOF	26	Laparostomy, cholecystectomy, necrosectomy, haemodialysis.

Table 2.7. **Details of deaths: NAC / SAMe Trial**

Index No.	Treatment group	Cause	Time from admission (days)	Clinical complication
Details of deaths attributable to acute pancreatitis (continued)				
8	Active treatment severe pancreatitis late presentation	Haemorrhage after necrosectomy, ARDS, IHD	65	Laparostomy and cholecystectomy with exploration CBD and necrosectomy.
Details of deaths not directly attributable to acute pancreatitis				
9	Active treatment severe pancreatitis early presentation	Cardiorespiratory arrest from tension pneumothorax	2	PM confirmed acute haemorrhagic pancreatitis with CBD stone and pneumothorax.
10	Active treatment severe pancreatitis early presentation	ARDS, sepsis	11 months after discharge	Second attack of pancreatitis 3 months after discharge; died 8 months later from ARDS, *staphylococcus* sepsis, and herpes from immuno-suppression for cardiac transplantation. PM showed further pancreatitis
11	Active treatment mild pancreatitis late presentation	Cancer of the prostate	37	Carcinomatosis found at laparotomy for pain, jaundice and persistent hyperamylasaemia.
12	Active treatment severe pancreatitis early presentation	Cerebral metastases	14 months after discharge	Bronchogenic carcinoma was diagnosed at the time of acute pancreatitis; palliative deep x-ray therapy.

Abbreviations: ARDS = adult respiratory distress syndrome, CABG = coronary artery bypass grafts, CVA = cerebral vascular accident, DIC = disseminated intra-vascular coagulation, ERCP = endoscopic retrograde cholangiopancreatography, GI = gastrointestinal, IHD = ischaemic heart disease, LV = left ventricular, MSOF = multisystem organ failure, PM = postmortem.

Table 2.7. (continued) **Details of deaths: NAC / SAMe Trial**

2.3.5. Blood micronutrient antioxidants

Table 2.1. (Individual Patient Data) reports individual, mean and median admission values for the antioxidants; serum selenium, plasma vitamins C and its reduced moiety ascorbic acid, serum vitamin E; β-carotene; and maker of free radical activity (%MR), for those patients where specimens were obtained at entry into the trial. Compared to historical laboratory control values the concentration of antioxidants were reduced and marker of free radical activity elevated, (Table 2.8) below.

	Serum selenium µg/l	**Plasma vitamin C mg/l**	**Ascorbate mg/l**	**Ratio reduced to oxidised Vitamin C**	**β-carotene µg/l**	**Vitamin E mg/l**	**%MR**
Lab controls median	119	13.7	11.0	0.78	88	10.9	2.18
range	81 - 161	5.3 - 18.5	5.2 - 15.8	0.37 - 2.62	19 - 254	5.7 - 15.5	0.81 - 3.9
All patients median	69	2.9	1.1	0.41	33	8.6	3.6
range	27 - 106	0 - 10.3	0 - 12.0	0.00 - 9.29	2 - 264	2.0 - 21.9	1.1 - 11.1
Wilcoxon rank sum test *(pts vs. cons)*	<0.0001	<0.0001	<0.0001	NS	<0.02	NS	<0.0001

Table 2.8 Antioxidant and markers of free radical activity in trial patients and historical laboratory (Lab) controls.

Table 2.2 reports the patient data divided into groups as determined by duration of symptoms at presentation, early (< 15 hours) and late (> 15 hours). No difference in blood antioxidant or markers of free radical activity levels was observed between these groups.

When the groups are divided into those managed with conventional supportive care (controls) and those that received the additional active therapy (active), Table 2.3, a difference between the groups was present for Vitamin E, median 9.3 mg/l controls, 6.9 mg/l active therapy, $p< 0.05$, Table 2.10. When divided by the predicted disease severity at time of entry, mild (APACHE II score <8) and severe (APACHE II score >7), of the biochemical measurements made it was only serum vitamin E levels was lower in the more severely ill individuals (median values 9.1 and 5.5 mg/l respectively, $p < 0.005$). Large differences were observed in the antioxidant level of both the mild or severe groups when compared to the historical laboratory controls, Table 2.10.

Biochemical measurement *Median (range)*	**Control / Conventional Rx**	**Active Rx**	**Mild**	**Severe**	**Lab controls**
Vitamin C mg/l	3.0 (0 - 10.3)	2.5 (0.3 - 9.8)	2.9 (0.5 - 10.3)	2.7 (0.3 - 8.9)	13.7 (5.3 - 18.5)
Selenium µg/l	69 (28 - 106)	64 (27 - 94)	72 (36 - 94)	64 (27 - 106)	119 (81 - 161)
Vitamin E mg/l	9.3 (5.3 - 21.9)	6.9 (2.0 - 15.2)	9.1 (5.5 - 21.9)	5.5 (2.0 - 15.2)	10.9 (5.7 - 15.5)
β-Carotene µg/l	39 (2 - 170)	30 (3 - 264)	35 (4 - 196)	28 (2 - 264)	88 (19 - 254)
%Molar ratio (9,11 / 9,12 LA)	4.16 (1.08 - 11.06)	3.34 (1.49 - 5.99)	3.54 (1.08 - 9.44)	4.09 (1.49 - 11.06)	2.18 (0.81 - 3.90)

Table 2.9. Blood antioxidant / micronutrient concentrations and marker of free radical activity at time of entry to study, divided into control and active therapy groups, and those with mild and severe disease.

Biochemical measurement	Conventional care vs. Active Rx	Mild vs. Severe	Mild vs. Lab controls	Severe vs. Lab controls
Vitamin C mg/l	NS	NS	< 0.001	< 0.001
Selenium µg/l	NS	NS	< 0.001	< 0.001
Vitamin E mg/l	< 0.05	< 0.005	< 0.001	NS
β-Carotene µg/l	NS	NS	< 0.05	< 0.001
%MR (9,11 / 9,12 LA)	0.010	NS	< 0.001	< 0.001

Table 2.10. Comparison of blood antioxidant and micronutrient concentration at time of entry to study against laboratory control values, Wilcoxon Rank Sum Test, p-values as shown.

2.3.6. Blood markers of free radical activity

Median %MR of 9, 11- to 9, 12-linoleic acid for the group of study patients as a whole were elevated compared to historical laboratory controls $p < 0.001$ (Table 2.8).

When the groups are divided into those managed with conventional supportive care (controls) and those that received the additional active therapy (Table 2.9 and 2.10) a difference between the groups was present for markers of free radical activity (percentage molar ratio), median 4.2% controls, 3.3% active therapy, $p = 0.01$. However, when the patients are divided into groups of mild and severe prognosis (Table 2.4), or early and late presentation (Table 2.2) no difference in the %MR was observed, Table 2.10.

In addition to the low plasma vitamin C levels observed in the patient group as a whole, analysis of specimens for ascorbic acid concentrations (the active reduced form of vitamin C), demonstrated that in acute pancreatitis patients 59% of the

plasma vitamin C present is in the oxidised form, compared to 22% in controls, Table 2.8.

These observation support the Manchester hypothesis of oxidative stress being implicated at the initiation-phase of pancreatitis.

2.4.0. Discussion

2.4.1. Re-examining the trial rationale

This randomised trial showed that, contrary to the expectation at the time the studies were planned (Braganza 1986b), treatment with SAMe and NAC for the first 24 hours from admission did not accelerate recovery or increase the salvage rate in patients with acute pancreatitis.

Since that time a substantial quantity of additional information has been published that rationalise these observations. Arguably the most important aspect of these are the interactions that occur between GSH and other antioxidant vitamins. In this regard it is worth noting that:

i) A synthetic ascorbic acid derivative ameliorates pancreatitis in the lethal dietary CDE model (Nonaka 1991 & 1992).

ii) Vitamin C and ascorbic acid were depleted in patients with acute pancreatitis, as reported elsewhere (Scott 1993, Bonham 1999).

iii) Analysis of the admission blood samples from patients in this trial, showed heightened free radical activity, sufficient to cause near-total oxidation of ascorbate, Table 2.8.

iv) Ascorbate inhibits inducible nitric oxide synthetase (*i*NOS) expression independent of NF-κB (Wu 2002).

v) Vitamin C closely interacts with GSH, buttressing supply of the latter during times of need (Winkler 1992, Mårtennsson 1993, Segal 1998).

vi) A similar benefit has been observed for the most effective booster of intracellular GSH, the monoethyl ester (Figure 2.2), in the caerulein model (Neuschwander-Tetri 1992).

vii) NAC ameliorates both caerulein and CDE-induced pancreatitis in mice (Demols 2000).

viii) Selenium is a requirement for the GSH reductase and peroxidase enzyme cycles (Stadtman 1990, Braganza 1991).

ix) Parenteral infusions of sodium selenite within 2 hours of the onset of symptoms resulted in a substantial decrease in mortality in an eastern German clinical study (Kuklinski 1991).

Redressing oxidative stress is thus a multifaceted task and thus on reflection it is perhaps unsurprising, that this trial which addressed just one aspect of this task (GSH availability) proved to be ineffective.

However, evidence continues to support published concepts from Manchester. Pancreastasis, from oxidative stress, seems to be the seminal event; a break-down in the methionine transsulphuration pathway appears to be causally linked to the exocytosis blockade; and diversion of proinflammatory free radical oxidation products into the bloodstream has now been documented, as has the inherently dysregulated nature of the inflammatory response (Braganza 1990, 1998). There is increasing agreement that an extreme degree of frustrated phagocytosis, and not wholesale premature activation of pancreatic zymogens, initiates the transformation to haemorrhagic pancreatitis and also MSOF (Braganza 1991). However, the secondary but rapid activation of zymogens compounds injury and accelerates death.

2.4.2. Glutathione and S-adenosylmethionine in signal transduction

Important information concerning the molecular events leading to exocytosis are described (Solner 1993, Wagner 1994). An N-ethylmaleimide sensitive factor (NSF) seems to be a critical requirement. It seems more than coincidence that GSH is an NSF (Schofield 1993), and that treatment with glutathione monoethyl ester (Figure 2.2) ameliorates pancreatitis in the caerulein model (Neuschwander-Tetri 1992), which shares many of the features of the human disease (Braganza 1990). The cysteine prodrug, L-2-oxothiazolodine-4-carboxylic acid (OTC) augments cysteine levels (Figure 2.2). Initially this compound was found not to do elevate GSH (Neuschwander-Tetri 1994), but subsequent reports challenge this (Lüthen 1997), and suggest that GSH may indeed be the NSF required for exocytosis (Lüthen 1994a).

It is worth reiterating that the decision to use SAMe in addition to NAC has been supported by the observation of benefit in the lethal allograft model of pancreatitis

(Scott 1992), and of methionine when given in conjunction with ascorbate in patients with relapsing pancreatitis (Bilton 1994). Depletion of SAMe in the acinar cell would be expected to compromise membrane fluidity for exocytosis (Hirata 1980) and to thwart the cell's strategy for refurbishing active phosphate groups (Timbrell 1982) and GSH (Sollner 1993) thereby triggering pancreastasis, Figure 2.2.

Previous studies had hinted at the importance of SAMe for pancreatic integrity. Animals reared on a choline-deficient ethionine-supplemented diet develop pancreastasis by 24 hours, by the next day casts indicating nonviable cells are present in the gland and hordes of leucocytes flood the interstitium. Death occurs by the fifth day, at a time when much of the elastase and phospholipase A_2 is in the active form, although the gland contains very little active trypsin (Lombardi 1975). An early burst of free radical activity has been shown by electron spin resonance studies (Nonaka 1989b). Free radicals may initiate degradation of IκB, the inhibitor of the transcription regulatory protein NF-κB. This liberates NF-κB which then migrates to the nucleus where it bind to the promotor and activates proinflammatory genes such as TNF-α and IL-6. Cytokine induction of this nature occurs early after initiation of experimental pancreatitis (Satoh 1999). NAC has been shown to inhibit such NF-κB activation (Backwell 1997) as has the antioxidant Pentoxifylline (Gomez-Cambronero 2000).

The weight of recent evidence thus argues for rapid treatment in pancreatitis, to restore the exocytosis pathway in the acinar cell and hints at the need for both SAMe and NAC. Yet this combination was unhelpful in this randomised clinical trial (Tables 2.5 and 2.6). Several interrelated factors may explain this anomaly:

- The time-lag to treatment was never < 4 hours and was sometimes > 240 hours (Table 2.6); animals models show how quickly the cascade of inflammatory changes occurs when exocytosis from pancreatic acinar cells is hampered (Braganza 1990 & 1995b). Indeed, *in vitro* studies have very recently shown that pancreatic elastase induces IκBbeta/IκBalpha complex degradation (within 30 minutes), NF-κB activation (within 60 minutes) and TNF-α expression (by 60

minutes) (Jaffray 2000). Giving NAC after 10 hours (which was the mean delay in presentation encountered in the 'early' group in the current study) is likely to be comparable to shutting the stable door well after the horse has bolted.

- The benefit from NAC treatment in our earlier experience (Figure 2.1) was associated with a longer duration of treatment (Braganza 1986b), as has also been shown in the setting of paracetamol poisoning (Keays 1991). The infusion of NAC and SAMe might have been more effective had the time period been prolonged.

- The proportion of patients with severe disease referred to our hospital was unusually high because it is a tertiary centre; dysequilibrium in oxidant-antioxidant, protease-antiprotease and immune-anti-immune systems are expected in such patents (Braganza 1993c & d).

Intermittent haemodialysis was routinely used in the earlier successful experience with NAC (Figure 2.1) because every patient had renal failure (Braganza 1986b). It is of interest that exchange transfusion was beneficial in a lethal animal modal of pancreatitis (Blower 1989). These manoeuvres would remove activated leukocytes or their cytotoxic products, which wreak much of the damage in acute pancreatitis (Braganza 1990, 1995b & 2000). The hypothesis that frustrated phagocytosis initiates MSOF (Rinderknecht 1988), in the same way as in fulminant sepsis (Braganza 1995b), is strongly endorsed by a study which showed that filters to remove neutrophils and platelets ameliorated in a model of severe pancreatitis (Kuroda 1994).

2.5.0. Conclusions

At the time that this trial was planned and started the methodology to measure GSH was not available to us. It was clearly necessary to find out whether the dose of NAC and SAMe administered had any impact on erythrocyte or plasma GSH and thiol concentrations. From three subsequent consecutive placebo-controlled studies

of antioxidant supplementation in patients with relapsing pancreatitis, it became apparent that a 'global' antioxidant supplementation was necessary, and that such combination should include ascorbate with methionine and selenium (Bilton 1994).

Antioxidant profiles from the patients in this trial support the need for a 'global' approach to treatment in acute as well as recurrent pancreatitis (Uden 1990a, Bilton 1994). Finally, it is of interest that such a combination has also been advocated, on clinical grounds, to reduce the risk of MSOF in patients with fulminant sepsis (Goode 1993).

2.6.0. Future studies

The inevitable time-lag to treatment of patients with acute pancreatitis in the UK, caused by the traditional referral route via primary care physicians, will prejudice the chance for success of any new treatment that appears promising in experimental models. The need for multi-centre studies is accepted, but raises logistic difficulties, not least to rectify adherence to present entry and physiological improvement criteria, as well as ascertaining the true cause of death.

Chapter 3

Folic acid and vitamin B_{12} status in pancreatitis

3.1.1. Introduction

The failure of parenteral SAMe and NAC to ameliorate the severity of acute pancreatitis, in the trial described in the previous chapter, despite the experimental evidence in support of such intervention, suggested that there may be other, as yet unrecognised factors, resulting in disruption of the methionine transsulphuration pathway.

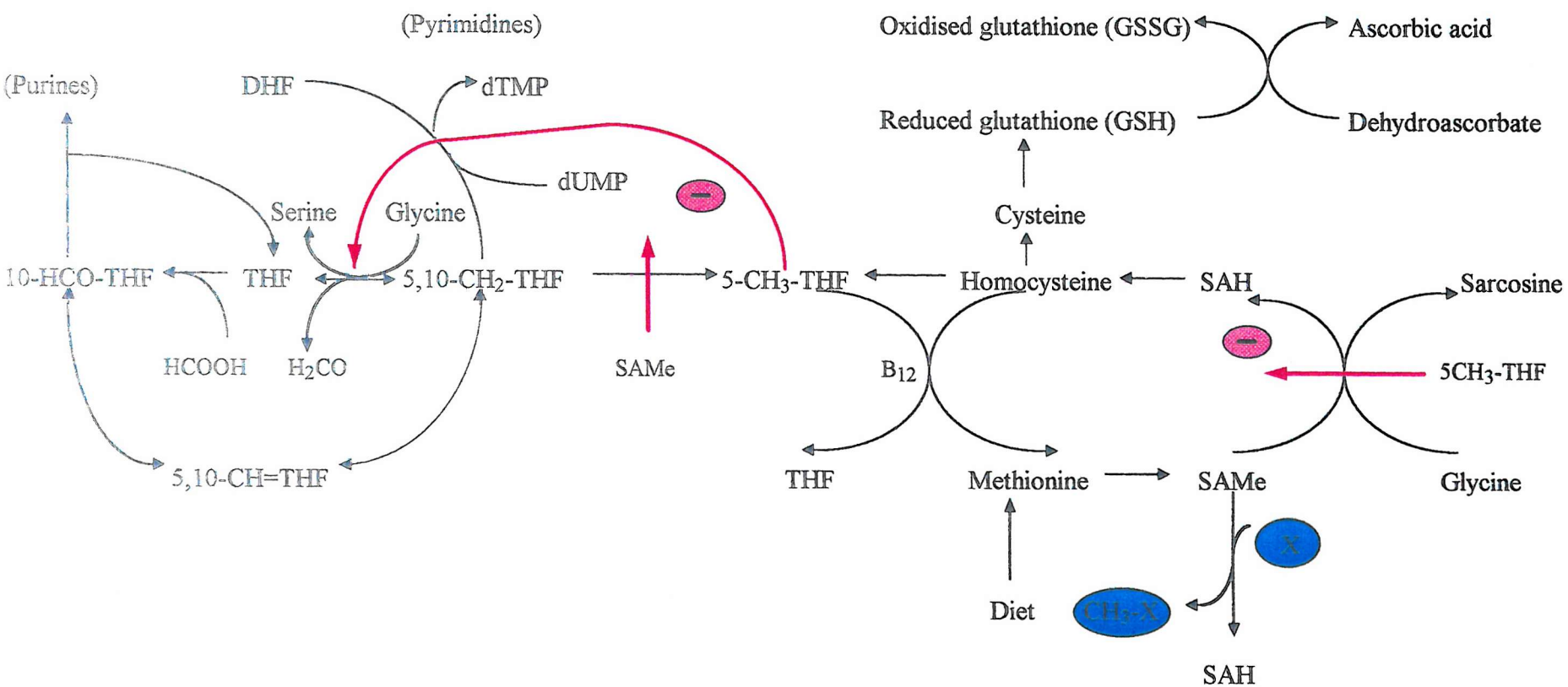
One-carbon metabolism is a vital limb of cellular metabolism. Folate is the principal carrier of one-carbon units and is central to nucleic acid production and both amino acid synthesis and degradation. Figure 3.1. demonstrates the state of our current understanding of folate metabolism. The mechanisms by which flux through the numerous folate dependant enzyme reactions are regulated, while the folate pool is maintained, are slowly being unraveled (Erbe 1975, Chanarin 1992). It is apparent that glycine N-methyltransferase (broken arrow below) avidly binds 5-methyl-tetrahydrofolate, but this folate moiety is not involved in the methyltransferase reaction that it catalyses (Wagner 1985). The abundance of glycine-N-methyltransferase within hepatocytes (up to 2% of cytosolic pool) and pancreatic acinar cells appears at first glance to be an extravagance, for the product, sarcosine, has no known metabolic function in mammals. This enzyme therefore accomplish a 'futile cycle' whose objective is to regulate the SAMe to SAH ratio. This ratio in turn controls and propels cellular methylation reactions. The reaction catalysed is:

Glycine + **S**-Adenosyl-L-**Me**thionine ➡ Sarcosine + **S**-Adenosyl-L-**H**omocysteine.

⇡

Glycine N-methyltransferase

Figure 3.1. **The role of glycine-N-methyltransferase in regulation of the methyl folate trap.**



Legend figure 3.1. *DHF* - dihydrofolate, *THF* - tetrahydrofolate, *5,10-CH=THF* - 5,10-methenyltetrahydrofolate, *5-CH_3-THF* - 5-methyltetrahydrofolate, *5,10-CH_2-THF* - 5,10-methylenetetrahydrofolate, *10-HCO-THF* - 10-formyltetrahydrofolate, *dUMP* - deoxyuridylate, *dTMP* - deoxythymidylate, *X* - represents a methyl acceptor, *SAMe* - S-adenosylmethionine, *SAH* - S-adenosylhomocysteine.
All forms of folate are in equilibrium except for *5-CH_3-THF* which is produced by the reduction of *5,10-CH_2-THF* in an irreversible reaction.
Red arrows indicate negative inhibition of enzyme by metabolic intermediate and blue ovals the transmethylation reactions.

After *Wagner et al.* 1985

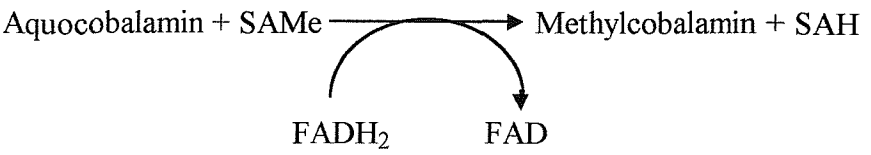
When purified, this enzyme has no detectable bound folate, but it is strongly inhibited by 5-methyltetrahydrofolate (Wagner 1985). How this observation relates to the methyl folate trap hypothesis is unclear, but one theory suggests that in times of methionine insufficiency (akin to B_{12} deficiency) folate is 'trapped' as 5-methyl-THF, this inhibits glycine-N-methyltransferase, which in turn preserves SAMe for onward use. The opposite ensues when methionine and hence SAMe is abundant. Here SAMe inhibits the reduction of 5,10-CH_2-THF to 5-CH_3-THF which in turn allows glycine-N-methyltransferase to dispose of excess SAMe, Figure 3.1.

Folate deficiency occurs in the face of dietary insufficiency, malabsorption states and with excess losses, usually through renal excretion. Alcoholism is often associated with multiple vitamin and other nutritional insufficiencies. Chronic alcohol ingestion results in folate deficiency (Russell 1983) which in turn impairs pancreatic acinar cell DNA synthesis (Elseweidy 1984). This might be one avenue by which alcoholism interferes with transsulphuration and precipitates an attack of pancreatitis. Whether anomalies of folate metabolism is a factor in pancreatitis associated with other aetiologies has not been examined.

Vitamin B_{12} is a co-factor in two enzymic reaction: methionine synthetase (5-methyl THF homocysteine methyltransferase) and methylmalonyl CoA mutase. Methionine synthetase utilises both folic acid and vitamin B_{12} in the following reactions:

1. Methylcobalamin + Homocysteine $\longrightarrow$ Methionine + reduced cobalamin (cob[1]alamin)
2. Cob[1]alamin + 5-methyl THF $\longrightarrow$ Methylcobalamin + THF

Although the Vitamin B_{12} in this transferase can catalyse many cycles of methyl transfer in this way, the prosthetic group occasionally reverts to the inactive 'aquo'-form, in which case it needs reactivating again. This reaction necessitates $FADH_2$ and SAMe in the following manner:



Methylmalonyl CoA mutase catalyses the reaction methylmalonyl CoA to succinyl CoA employing the Vitamin B_{12} derivative 5'-deoxyadenosylcobalamin. This reaction permits the utilisation of propionyl CoA produced in the catabolism of branched chain amino acids and odd chain length fatty acids. Without this capacity, significant amounts of potential energy are lost and genetic anomalies of methylmalonyl CoA mutase activity result in failure-to-thrive, mental deterioration and acidosis (Stokke 1967).

Figure 3.1. shows the intricate relationship between folic acid, Vitamin B_{12}, homocysteine, glutathione and Vitamin C. In addition to the links between folic acid and Vitamin B_{12}, an interrelationship may exist between ascorbic acid and Vitamin B_{12} metabolism (Kahn 1968) and ascorbic acid with labile folate derivatives (Wilson 1983). Furthermore, ascorbic acid is utilised in the reduction of oxidised glutathione (Winkler 1992) and redundancy or interchangeability has been demonstrated to exist between GSH and ascorbate, in that GSH may avert scurvy in guinea-pigs (Mårtensson 1993). As ascorbic acid deficiency is reported in acute pancreatitis (Scott 1994, Bonham 1999) and its inclusion is importance in the antioxidant supplement cocktail used with benefit in the therapy of pancreatitis (Uden 1990a, Bilton 1994), we undertook to look at folic acid and Vitamin B_{12} levels in pancreatitis and relate those to Vitamin C and glutathione. For, if deficiencies were identified, these would potentially offer easily amenable avenues, by which transsulphuration could be supported during an attack and through which subsequent prophylaxis against pancreatitis might prove possible. Thus, the next phase of this thesis was to quantitate these vitamin in the blood of patients with acute and chronic pancreatitis.

3.2.0. Materials and Methods

3.2.1. Patients

Consecutive patients admitted to Manchester Royal Infirmary, diagnosed as having acute pancreatitis on the conventional criteria of a compatible history and a serum amylase greater than three times the upper limit of normal were invited to enter the study. Disease severity as predicted by the APACHE II score (with a score > 7 considered severe, Bradley 1993), was recorded at the time of diagnosis, as was the time that had elapsed between the first symptom being experienced and admission to the study. 16ml peripheral venous blood was taken as soon as possible once the diagnosis had been established, for the simultaneous estimation of serum Vitamin B_{12}, serum and erythrocyte folate, plasma Vitamin C and plasma and whole blood glutathione. Results were compared with historical laboratory controls.

The Vitamin B_{12}, Vitamin C and folic acid status of the subjects entered into the challenge studies, reported in the following chapter, were also documented. These subjects comprise three groups, healthy controls and patients with a past history of recurrent acute (non-gallstone) and chronic pancreatitis. In the individuals with a history of pancreatitis, levels were performed during the quiescent phase of their disease, at the time of entry into the challenge studies. Again, results were compared to historical laboratory controls. In the case of the challenge study group with quiescent recurrent acute pancreatitis comparisons were also made with the patients with active acute pancreatitis. Plasma and whole blood GSH levels in the challenge study subjects were the average of fasting baseline levels observed in phase 1 and 2 of these studies (see Chapter 4).

A 24 hour urine collection was attempted in patients presenting with acute pancreatitis in an attempt to quantitate urinary folic acid loss during the initial stages of an attack.

3.2.2. Biochemical assays

Admission blood samples for glutathione estimation were processed immediately to prevent auto-oxidation of glutathione. GSH was measured by kinetic enzymic assay, Appendix A. Total Vitamin C was quantitated by the spectrophotometric method in meta-phosphoric acid-titrated plasma, Appendix A. The Vitamin C metabolites; ascorbic acid, dehydroascorbic acid and diketogulonic acid were not quantitated separately, as this aspect had already been addressed in the biochemical protocol of the NAC / SAMe trial (Chapter 2). Serum and erythrocyte folic acid were measured by microbiological assay with chloramphenicol-resistant *lactobaccilus casei* (Chanarin 1972). It of interest that this assay utilises added ascorbic acid to prevent air oxidation of reduced and polyglutamate folate moieties (Wilson 1983). Vitamin B_{12} in serum was measured by a standard laboratory method based on radio-dilution (Matthews 1967).

3.2.3. Statistical analysis

Comparison of the results obtained for folic acid, Vitamin B_{12}, Vitamin C and glutathione with historical laboratory normal ranges were performed using the non-parametric Wilcoxon Rank Sum Test to avoid distributional assumptions. Correlation coefficient between groups were calculated by the Spearman method. The null hypothesis was denied when p-value < 0.05.

3.2.4. Ethical Approval

These investigations were passed by the Ethics Committee of The Central Manchester Healthcare Trust. Informed consent was obtained from patients, after the background to the studies had been explained. They were informed, that in addition to blood tests indicated for the clinical management of their disease, that an additional volume (16ml) would be required, for analysis of vitamins and another chemicals, produced under normal circumstances, by the body. The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki.

3.3.0. Results

3.3.1. Acute pancreatitis patients

24 patients with acute pancreatitis were entered into the study over a period of 18 months. Their demographic data is reported in Table 3.1. Eleven were male, 13 female, with an average age of 45.6 years. The aetiology of acute pancreatitis were gallstones (10), alcohol (6) and in 8 patients the cause was unknown. Four patients had prognostically severe disease at the time of admission as predicted by an APACHE II score > 7. The mean APACHE II score for the group as a whole was 4.25 (1 s.d. +/- 4.69); median = 3. The average duration of symptoms prior to admission was 35.6 hours (1 s.d. +/- 34.4); median = 25.5. Acute pancreatitis resulted in the death of patients number 3, 6 and 17.

Serum Vitamin B_{12} values in patients with acute pancreatitis were generally within the reference range; patients median, (range) 365μmol/l, (69 - >1000); controls 270ng/l, (118 - 645), no significant difference. By contrast, serum and RBC folate ($p < 0.001$), plasma ($p < 0.001$) and whole blood glutathione ($p < 0.01$), and plasma vitamin C levels ($p < 0.001$), were lower in admission blood samples from the patients when compared to historical controls, Tables 3.2 and 3.3.

Subgroup analysis, revealed that the plasma Vitamin C levels were lower in the 6 patients with alcoholic pancreatitis than in the other 18 patients, (median, range) 2.95, 1.3 - 4.5 versus 4.7, 1.1 - 11.6 mg/l, $p < 0.05$. No differences were observed for Vitamin B_{12}, folate or GSH in the alcoholic, nor in any of the analytes in the gallstone sub-group.

Despite low plasma and RBC folate levels in the acute pancreatitis group as a whole, only two individuals (both with heavy alcohol intakes) had an MCV above the normal range. A significant positive correlation is seen, in the patients with acute pancreatitis, when serum folate concentrations are compared to those of plasma vitamin C, ($T = 2.78$; $p < 0.01$), Figure 3.2. No significant correlation was observed between plasma Vitamin C and RBC folate, or either of these vitamins and plasma or whole blood GSH.

Table 3.1 Demographic and clinical details of patients with acute pancreatitis

Patient No.	Gender	Age	Aetiology	Smoking history (pk yrs)	Alcohol intake units / w	Time from 1st symptom to admission (hr.)	Admission APACHE II score
1	M	45	Idiopathic	0	14	<6	2
2	F	33	Idiopathic	0	0	6	0
3†	F	38	Gallstones	16	5	96	10
4	M	50	Idiopathic	0	20	27	3
5	M	39	Alcohol	41	80	24	0
6†	M	46	Alcohol	14	420	33	5
7	F	26	Idiopathic	0	0	20	5
8	M	59	Gallstones	40	30	36	6
9	M	40	Idio/Alcoh	7	40	18	5
10	M	76	Gallstones	48	2	10	14
11	M	36	Alcohol	8	10	34	0
12	F	21	Gallstones	0	2	48	0
13	F	24	Gallstones	0	0	48	0
14	F	47	Alcohol	16	50+	>12	2
15	M	57	Gallstones	33	0	48	9
16	F	23	Gallstones	0	10	96	0
17†	M	88	Idiopathic	0	0	54	18
18	M	49	Alcohol	38	80+	15	3
19	F	26	Gallstones	7	5	6	1
20	F	43	Idiopathic	0	0	9	0
21	F	55	Idiopathic	Ex	1	5	6
22	F	49	Idiopathic	22	4	11	2
23	F	65	Gallstones	0	2	48	5
24	F	65	Gallstones	0	0	144	6

Table 3.2 Haematology, vitamin and GSH data of patients with acute pancreatitis

Patient No.	Hb (g/dl)	MCV (fl)	Serum B_{12} (ng/l)	Serum folate (μg/l)	RBC folate (μg/l)	Plasma Vitamin C (mg/l)	Plasma GSH (μmol/l)	WB GSH (μmol / g Hb)
1	13.4	82	485	4.6	311	11.6	4.83	6.81
2	12.6	75	282	4.1	333	6.4	2.87	11.27
3†	8.4	81	399	3.9	346	2.8	4.09	8.07
4	14.6	82	673	15.0	393	4.8	1.63	6.45
5	15.1	87	217	3.9	291	3.2	2.63	8.04
6†	14.6	85	261	14.2	359	3.8	3.45	8.63
7	13.0	78	288	3.9	288	5.0	3.97	7.67
8	11.9	83	389	4.6	355	2.2	1.19	7.22
9	15.7	88	168	4.0	438	1.3	1.74	8.82
10	13.2	88	500	4.9	355	4.3	1.94	10.88
11	13.2	91	722	2.0	151	2.7	1.38	10.65
12	15.8	82	495	6.5	361	8.6	2.73	9.11
13	13.3	84	296	7.4	357	9.7	1.7	7.09
14	13.8	105	281	5.2	247	4.5	2.12	6.26
15	13.6	82	292	2.3	325	1.1	3.32	9.48
16	13.9	90	430	10.2	331	7.3	3.39	7.22
17†	17.5	88	153	3.3	312	5.3	3.83	8.00
18	14.9	99	189	<1.0	245	2.1	2.25	9.94
19	13.0	85	413	10.0	437	2.9	2.00	8.84
20	11.9	74	69	10.9	366	8.3	5.61	7.55
21	14.3	87	340	12.2	396	4.6	2.79	7.66
22	10.7	87	559	3.4	573	1.9	2.48	7.21
23	10.9	87	>1000	4.2	368	4.5	1.06	9.43
24	10.6	82	838	3.5	363	4.3	0.95	7.12

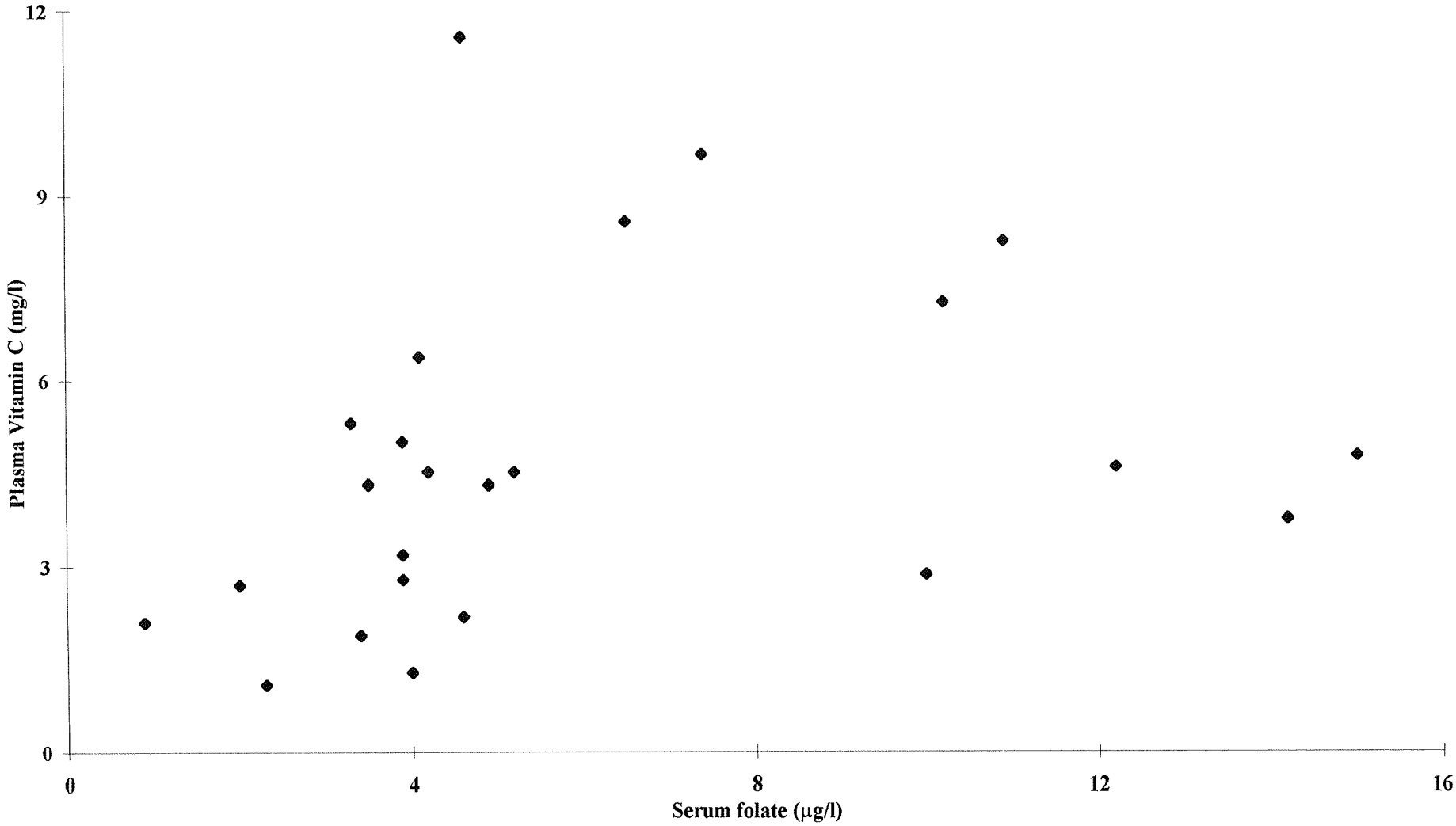
Table 3.3. Folate, Vitamins B_{12} & C and GSH results with comparisons between controls, AP and quiescent RAP.

	(A) Historical Controls (n = 30 [a]; n = 18 [b])	(B) Acute Pancreatitis (n = 24) probability vs. (A)	(C) Challenge study RAP (n = 9 [a]; n = 8 [b]) probability vs. (A) & (B)
Serum Folate (µg/l)	8.1 [a] (1.8 - 18.1)	4.6 (<1.0 - 39) P < 0.001	5.2 [a] (2.0 - 11.5) P < 0.05 & NS
RBC Folate (µg/l)	500 [a] (220 - 894)	355 (151 - 573) P < 0.001	320 [a] (292 - 619) NS & NS
Serum B12 (ng/l)	270 [a] (118 - 645)	365 (69 - >1000) NS	312 [a] (217 - 474) NS & NS
Plasma Vitamin C (mg/l)	14.2 [a] (6.3 - 19.0)	4.4 (1.1 - 11.6) P < 0.001	5.1 [a] (4.3 - 15.5) P < 0.002 & P < 0.05
Whole Blood GSH (µmol/g HB)	10.14 [b] (7.49 - 12.21-)	8.02 (6.21 - 11.27) P < 0.01	8.67 [a] (6.66 - 9.12) P < 0.005 & NS
Plasma Total GSH (µmol/l)	5.75 [b] (3.90 - 8.30)	2.56 (0.95 - 5.61) P < 0.001	4.57 [b] (3.34 - 6.72) NS & P < 0.005

Data expressed as median (range). Wilcoxon rank sum probability of acute pancreatitis versus historical controls and challenge study RAP versus acute pancreatitis group.

NS - no significant difference.

Figure 3.2. **Serum folate versus plasma Vitamin C concentrations in acute pancreatitis**



3.3.2. Challenge study patients

Table 3.4 reports the demographic details of the challenge study subjects. These are described in greater detail in Chapter 4. Fasting blood values for Vitamins B_{12} and C, folic acid and glutathione in these individuals are reported in Table 3.5. Statistical comparisons of these antioxidants levels, between groups are summarised in Table 3.6. When compared with historical laboratory controls, Vitamin B_{12} and plasma GSH levels were similar in all groups. RBC folate in the 'challenge study' controls differed from the historical laboratory controls ($P< 0.05$), but the group was small ($n = 5$) and thus, may be subject to type 2 error.

When the two 'challenge study' pancreatitis groups are examined independently, similar differences to the control group are observed. Of particular note is the apparent low level of whole blood glutathione in patients with a history of RAP ($p < 0.005$) and CP ($p < 0.05$) compared to historical controls. However, this difference was not confirmed, when GSH values were compared to the challenge studies control subjects. Similarly, folic acid levels were reduced in patients with a history of RAP compared to historical controls ($p < 0.05$).

When the results of challenge study subjects with a history of pancreatitis, both recurrent acute or chronic, are combined into a single group (allpanc), plasma vitamin C ($p < 0.001$) and whole blood GSH ($p < 0.001$) concentrations were very low, while RBC folate was reduced, but not to such a great extent ($p < 0.02$), compared to historical controls, Table 3.6.

Finally, comparison of the two groups of patients with acute pancreatitis: those with an active acute attack and those from the challenge studies with quiescent recurrent acute pancreatitis reveals some interesting observations. Serum vitamin B_{12} levels did not differ. Serum folate did not differ between the patient groups, but was lower in both compared to the historical laboratory control group. Plasma vitamin C was reduced in both groups compared to controls, with those suffering from an acute attack being significantly lower than those between bouts. Plasma GSH

results were similar to those for vitamin C, both patient groups had low values with actual concentrations being lower in active than quiescent disease, Table 3.3.

3.3.3. Urinary folate excretion

We attempted to explore the possibility that folate is lost through excess renal loss in AP, but found that urinary folate concentrations at the time of admission, were below the limit of detection of our microbiological assay in many samples. 24 hour collections suffered from stability problems, especially in catheter stream samples. As a result of these problems, inadequate data was obtained of this aspect and the results are not reported here.

Table 3.4 Demographic and clinical details of challenge study subjects (from Chapter 4)

Subject initials	Gender	Age	Pancreatic morphology (aetiology - exocrine status)	Smoking history (pack yrs)	Alcohol intake - units/week
HBS	M	50	normal	nil	10
PFN	F	62	normal	nil	25
EGrY	F	35	normal	6	15
MHN	M	50	normal	nil	40
AJN	M	37	normal	nil	nil
GKY	M	52	normal	20	nil
NST	M	20	normal	4	60
OSN	F	60	normal	2	3
RMcE	M	39	RAP (alcohol)	31	Ex - 200
EGY	F	65	RAP (gallstone)	24	2
DHD	M	54	RAP (alcohol)	20	Ex - 140
PHS	M	42	RAP (idiopathic)	5	30
CKN	F	25	RAP (pre- & post cholecystectomy)	Nil	Nil
EMcE	F	32	RAP (gallstone & transient pseudocyst)	8.5	21
JMK	M	32	RAP (alcohol)	14	80
SWS	M	31	RAP (alcohol)	9	60
PFH	F	61	RAP (gallstone)	17	Nil
JCN	M	44	CCP & pancreatic cysts (alcohol - PI)	28	Ex - 120
JGH	M	49	CCP & portal vein thrombosis (alcohol - PS)	30	Ex - 300
NHN	M	24	NCCP (idiopathic - PS)	10	Nil
LJS	F	23	CCP (alcohol - PS)	1.4	25
GMN	F	83	NCCP (idiopathic - PI)	Nil	10
POE	M	32	CCP & intermittent jaundice (alcohol - PI)	15	Ex- 120
EPY	M	46	NCCP & pseudocyst (alcohol - PS)	20	Ex - 55

(N)CCP - (Non)-Calcific chronic pancreatitis. PI - Exocrine insufficient
PS - Exocrine sufficient. RAP - Recurrent acute pancreatitis

Table 3.5. Haematology, vitamin and GSH data of challenge study subjects

Subject initials	Hb (g/dl)	Serum B_{12} (ng/l)	Serum folate (μg/l)	RBC folate (μg/l)	Plasma Vitamin C (mg/l)	Plasma GSH (μmol/l)	WB GSH (μmol/g Hb)
HBS	13.9	243	5.4	274	3.8	4.78	9.97
PFN	11.7	284	18		21.7	6.13	12.19
EGrY	12.2	272	6.4	398	14.4	6.42	9.31
MHN	12.8	353	18		24.3	4.54	8.70
AJN	15.8	348	4.9	247	11.7	5.12	6.01
GKY	14.4	526	10.3	315	16.2	4.68	8.06
NST	15.0	342	11	470	12.1	11.95	8.23
OSN	13.4				6.3	4.87	10.30
RMcE	14.9	217	3.9	296	4.4	6.14	7.82
EGY	12.6	230	11.5	619	7.7	3.62	8.67
DHD	14.1	396	6.4	416	10.4	3.34	8.12
PHS	13.1	351	5.2	320	5.1		8.91
CKN	12.1	260	7.3	435	5.1	4.92	6.66
EMcE	13.1	312	2	292	4.6	6.72	8.95
JMK	15.8	244	4.5	455	12.6	5.26	6.85
SWS	14.6	474	2	308	4.3	4.22	9.12
PFH	13.6	465	7.5	317	15.5	4.00	8.85
JCN	14.6	581	5.9	427	5.8	3.18	9.79
JGH	13.0	253	7.7	598	4.7	5.17	8.66
NHN	15.3	827	5	414	7.2		6.23
LJS	12.5	260	6.4	405	7.9	5.39	10.89
GMN	10.9	341	18		2.6	6.62	7.81
POE	13.5	249	1.1	363	5.2	1.59	7.11
EPY	13.9	343	11	311	9.6	3.59	

Table 3.6 Folate, Vitamins B_{12} & C and GSH results with comparisons between controls and challenge study patients with quiescent RAP and CP.

	Historical controls (HC)	Challenge study controls (CSC)	Challenge study RAP	Challenge study CP	Challenge study all pancreatitis
Serum Folate (μg/l)	8.1 [30] (1.8 - 18.1)	10.3 [7] (4.9 - 18.0) *NS*	5.2 [9] (2.0 - 11.5) *P < 0.05 & NS*	6.2 [7] (1.1 - 18.0) *NS & NS*	6.2 [16] (1.1 - 18.0) *NS & NS*
RBC Folate (μg/l)	500 [30] (220 - 894)	315 [5] (247 - 470) *P < 0.05*	320 [9] (292 - 619) *P < 0.05 & NS*	410 [6] (311 - 598) *NS & NS*	405 [15] (292 - 619) *P < 0.02 & NS*
Serum B12 (ng/l)	270 [30] (118 - 645)	342 [7] (243 - 526) *NS*	312 [9] (217 - 474) *NS & NS*	341 [7] (249 - 827) *NS & NS*	327 [16] (217 - 827) *NS & NS*
Plasma Vitamin C (mg/l)	14.2 [30] (6.3 - 19.0)	13.3 [8] (3.8 - 24.3) *NS*	5.1 [9] (4.3 - 15.5) *P < 0.002 & NS*	5.8 [7] (2.6 - 9.6) *P < 0.001 & < 0.05*	5.5 [16] (2.6 - 15.5) *P < 0.001 & P < 0.05*
Whole Blood GSH (μmol/g Hb)	10.14 [18] (7.49 - 12.21)	9.01 [8] (6.01 - 12.19) *NS*	8.67 [9] (6.66 - 9.12) *P < 0.005 & NS*	8.42 [6] (6.23 - 10.89) *P < 0.05 & NS*	8.66 [15] (6.23 - 10.89) *P < 0.001 & NS*
Plasma Total GSH (μmol/l)	5.75 [18] (3.90 - 8.30)	4.99 [8] (4.54 - 11.95) *NS*	4.57 [8] (3.34 - 6.72) *NS & NS*	4.38 [6] (1.59 - 6.62) *NS & NS*	4.57 [14] (1.59 - 6.72) *NS & NS*

Data expressed as median (range), with n-value as superscript.
Wilcoxon Rank Sum Test probability versus historical controls **(HC)** and challenge study controls **(CSC)**.

NS. No significant difference.

3.4.0. Discussion

This study identifies low blood folate levels at the time of admission with acute pancreatitis and its persistence (at least in serum) during the quiescent phase between attacks, Table 3.3. Folate deficiency can result from a reduced premorbid intake, heightened utilisation or excessive loss. One explanation for the low erythrocyte and serum folate concentrations, at the time of admission in patients with acute pancreatitis, is inadequacy of the pre-morbid diet. A similar conclusion was reached in previous studies of vitamin C levels in pancreatitis (Scott 1994, Bonham 1999). It is noteworthy that the major food sources of vitamin C (citrus fruits) and folic acid (green leaf vegetables) have little overlap. Dietary histories often reveal poor intake of one of these groups is associated with a similar paucity of the other. Even if intake proves adequate, one study has supported reduced folic acid absorption from the intestines of patients with chronic pancreatitis (Mossner 1986). Urinary folate excretion rate is increased by 20 - 40% with chronic alcohol ingestion (Russell 1983). We attempted to explore whether this route of excretion is relevant in patients with acute pancreatitis, but for reasons discussed insufficient data was obtained to analyse. This remains an area where future work to quantify urinary folate losses in acute pancreatitis is indicated.

Pancreatic exocrine insufficiency appears to explain some isolated cases of Vitamin B_{12} malabsorption (LeBauer 1968), but there was no relationship to pancreatic exocrine status in the present study, for Vitamin B_{12} concentrations in patients with pancreatic insufficiency did not differ to those with preserved function. It is possible that the normal serum Vitamin B_{12} concentrations observed, falsely reflect depleted tissue availability. In chronic alcoholic liver disease, Vitamin B_{12} leaks into plasma where elevated levels of holo-transcobalamin I and transcobalamin III prevent blood Vitamin B_{12} from being excreted despite high levels (Baker 1998). Oxidation of methylcobalamin has been described, for it is well established that the anaesthetic / analgesic agent nitrous oxide (N_2O) causes megaloblastic anaemia through inhibition of methionine synthetase (Deacon 1980, Nunn 1982) and even subacute combined degeneration of the cord (Layzer 1978). Reduced uptake of

circulating folate by peripheral organs, with the subsequent urinary loss of excessive amounts of folate, associated with a co-existing lack of circulating Vitamin C, has been demonstrated in rats exposed to nitrous oxide (Lumb 1982). N_2O by oxidising methyl cobalamin, inactivates methionine synthetase, at an inhaled level of 5400 ppm (Sharer 1983). One metabolic response to this is the induction of betaine-homocysteine methyltransferase the other hepatic enzyme capable of recycling homocysteine to methionine (Lumb 1983).

One priority of intermediate metabolism is to guarantee an unrestricted supply of methyl groups, for the diverse array of homeostatic and regulatory processes that require them. The lethal effect of only 5 days of the CDE diet in mice examplifies this point, Figure 3.3. Methyl group insufficiency restricts its availability and as a result impairs the recycling of homocysteine. This results in a rise in the level of SAH, although SAMe concentration are preserved (Cook 1989).

Whatever the explanation for the low folate levels identified in this study, the sequelae of dietary folate deficiency in rats may result in a progressive accumulation of SAH, despite a compensatory induction of the alternative choline-betaine methyltransferase pathway (Allen 1993). Indeed, after 5 weeks on a folate deficient diet SAM : SAH ratio in rat pancreas fell from 7.48 to 0.68 (Balaghi 1991), implying that utilisation of methyl groups from the universal donor, SAMe, has outstripped the *de novo* supply and sources available by the recycling of homocysteine. *In vitro*, ethionine inhibits amylase release from AR42J pancreatic cell lines and results in a rise of SAH levels, again suggesting that there is a failure in the recycling of SAH (Capdevila 1997). It is hardly surprising therefore, that SAMe itself proved protective in a post-transplant immuno-suppression model of experimental pancreatitis (Scott 1992).

The combination of folic acid, Vitamin C and glutathione deficiencies, seen in our acute pancreatitis and challenge study patients, supports the experimental evidence that pancreatic acinar cell injury with impedance to exocytosis, or pancreatitis may result from a lack of:

a) methionine (Véghelyi 1950)
b) folate (Wagner 1985, Balaghi 1995)
c) depletion of pancreatic thiols (Dabrowski 1990, Neuschwander-Tetri 1992)

It also rationalises why pre-treatment with a synthetic analogue of ascorbate (Nonaka 1991) or intracellular thiols (Neuschwander-Tetri 1992) ameliorate pancreatic damage in differing experimental models. Similarly, methionine supplementation limits alcohol-induced liver damage in rats (Parlesak 1998).

Critical illness has been associated with acute folate deficiency (Ibbotson 1975, Geerlings 1997), despite the finding of blood levels *within* the normal range. This is not an isolated situation for homocysteine, methylmalonic acid, 2-methylcitric acid and cystathionine levels were reduced by supplementation of folate, Vitamin B_6 and B_{12}, in elderly patients who apparently had normal levels of these blood vitamin; again implying a poor correlation between blood and tissue concentrations (Naurath 1995). This lack of correlation has also been noted between plasma and erythrocyte folate in an elderly Canadian population (Quinn 1996) and between blood (plasma / erythrocyte) and tissue (sigmoid colon) folate levels (Meenen 1996). Meta-analysis supports the benefit of folic acid and Vitamin B_{12} supplementation in reducing blood homocysteine levels in the general population (Homocysteine Lowering Trialists' Collaboration 1998). This implies that the 'normal' population has a suboptimal capacity to recycle methionine from homocysteine, despite apparently adequate co-factor availability. Thus plasma homocysteine concentrations have been show to be a sensitive functional indicator of the intracellular availability of cofactors folate, Vitamin B_{12} and B_6 (pyridoxine), (Cravo 1996).

Is there an intracellular explanation for how these observations translate into models involved in the aetiology of pancreatitis? Oxidative stress disrupts the SNAP-SNARE vesicle trafficking system. Such a disruption may be due solely to reduced methylation of a transport G-protein (Capdevila 1997). Thus any process interfering with SAMe availability might cause this. Additionally, it has been suggested that either glutathione itself or a glutathione-like molecule plays a central

role in regulated trafficking (Stenson 1983). The resulting disruption to polarised exocytosis, from methyl group depletion, is followed by a discharge of pro-inflammatory free radical oxidation products, via the baso-lateral membrane rather than from the apex, into the pancreatic interstitium heralding an acute attack of pancreatitis (Braganza 1995b).

Hyperhomocysteinaemia is an independent risk factor for all cardiovascular (Nygård 1997) and cerebrovascular diseases (Perry 1995). The benefit of reducing the endothelial dysfunction precipitated by hyperhomocysteinaemia has been observed with both folate (Usui 1999) and an antioxidant vitamin supplementation (Nappo 1999). Indeed, there are two papers suggesting an association between pancreatitis and vascular disease (Gullo 1982 & 1996). To date very little data has been published on plasma homocysteine levels in pancreatic disease and further studies, which proved to be not possible during the course of this work, are required in this area in future.

In conclusion, low levels of folate in AP, that persists between attacks, coupled with a lack of Vitamin C, could explain why replenishment of thiols with only SAMe / NAC did not ameliorate disease in the previous chapter. Secondly, that hyperhomocysteinaemia would be predicted under these circumstances and would be amplified, should ongoing oxidative stress incapacitate vitamin B_6-dependant enzymes, e.g. cystathionine β-synthetase (Davies 1986), Figure 3.3. Supporting the need to replenish antioxidant defences, from several angles in pathologies in which oxidative stress has been implicated in the pathogenesis.

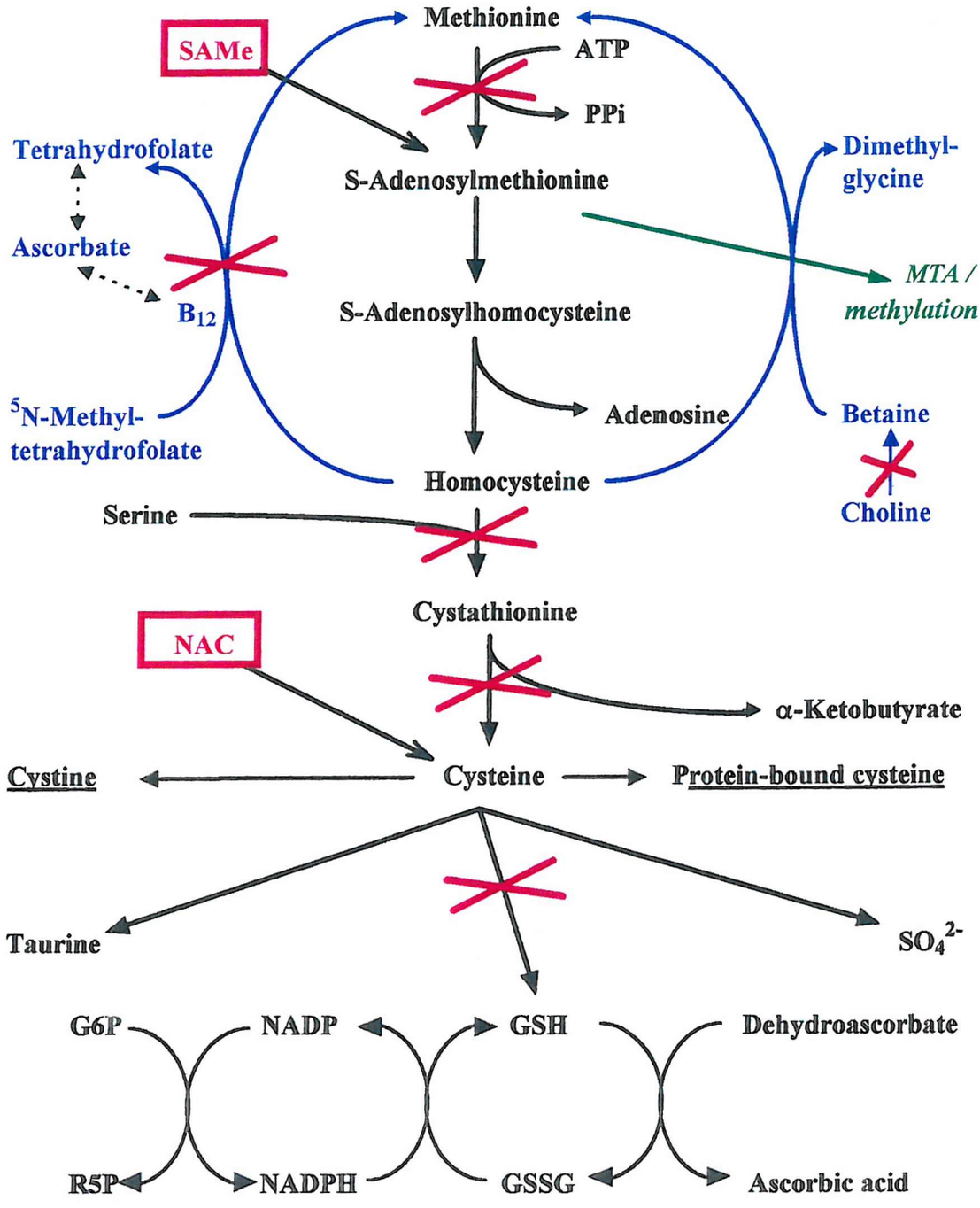


Figure 3.3. **Methionine transsulphuration pathway and the sites of 'blockade' induced by CDE diet, oxidative stress and folate deficiencies.**

Key to Figure 3.3.

Red crosses (solid)		Sites where transsulphuration pathway is interrupted by the CDE diet and oxidative stress.
Red boxes (solid)		Negative allosteric feedback inhibition of enzyme by metabolic intermediate.
Green arrows (solid)		Transmethylation 'output' of methyl groups.
Blue lines (solid)		Pathways that recycle homocysteine to methionine

Abbreviations:		
	THF	tetrahydrofolate
	G6P	glucose 6 phosphate
	6PG	6 phosphogluconate
	NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
	GSH	glutathione
	GSSG	oxidised glutathione
	SO_4^{2-}	inorganic sulphate

Chapter 4
Sulphur Amino Acid Challenge Studies

4.1.0 Philosophy

Chapter 1 reviewed the evidence that methionine metabolism is disturbed in patients with pancreatic disease. To recap, ethionine, a potent methionine antagonist causes pancreatitis in many species. The pancreatic lesions produced vary from severe pancreatitis with haemorrhagic necrosis to atrophy of the gland depending on the dosing regime (Kahn 1959). This effect of ethionine on acinar cells is prevented by the co-administration of methionine but not by its metabolites cysteine or choline (Farber 1950). These observations suggest that a disturbance high up in the transsulphuration pathway may cause pancreatitis, Figure 1.3. Interruption of the first enzymic step of this pathway, that catalysed by methionine adenosyltransferase, will limit SAMe synthesis. SAMe deficiency alters intracellular energy charge and limits regulatory methylation processes. It is probable that these regulatory methylation reactions are necessary for effective trafficking and fusion of zymogen granules with the acinar cell apical membranes.

Aberrant methionine metabolism in acute pancreatitis is supported by the observations of Roth *et al.* 1985. This paper reported that plasma methionine was elevated in AP when accompanied by organ failure, compared to normal individuals. In contrast, the concentration of most other amino acids fell. However, cystathionine concentrations were also higher suggesting that any disturbance to the transsulphuration pathway is not restricted solely to the first reaction of the pathway, Figure 1.3.

Further evidence of disturbed methionine metabolism came the following year from Sweden. Patients with alcohol-induced chronic relapsing pancreatitis were found to have higher levels of both methionine and cystathionine in their leukocytes and urine compared to controls or patients undergoing routine cholecystectomy (Märtensson 1986). The authors also reported significant depression of leukocyte GSH

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concentrations. GSH is an 'end product' of transsulphuration and this observation reinforces the notion that the integrity of methionine metabolism is interrupted in pancreatitis. Indeed, caerulein-induced pancreatitis in mice is associated with a precipitous drop in total pancreatic GSH and administration of glutathione monoethyl ester reduces this fall and ameliorates the severity of the pancreatitis (Neuschwander-Tetri 1992).

Work from our own laboratories suggests that plasma SAMe levels are below normal in patients with pancreatic disease between attacks, but that they rise at the time of relapse (Schofield 1991, Uden 1992). Again, this suggests that disturbances in transsulphuration in AP affect enzymic steps other than the first, for were this to be the case, an isolated elevation in plasma methionine and a fall in all subsequent intermediates would be expected.

In the preceding chapters I have reported low blood GSH and folic acid levels in patients with acute pancreatitis. Folate deficiency compromises the re-methylation of homocysteine to methionine by inhibiting methionine synthetase activity, Figure 3.1.1. A folate-deficient diet inhibits amylase secretion from perfused duodenal segments and results in pancreatic oedema in anaesthetised rats (Balaghi 1995). Vitamin B_{12}, also a cofactor of methionine synthetase, has been reported to be deficient due to malabsorption in pancreatic insufficiency (LeBauer 1968). However, this lack is rare in chronic pancreatitis and does not depend upon degree of exocrine insufficiency (Glasbrenner 1991). Furthermore, we did not identify B_{12} insufficiency in acute pancreatitis patients (Chapter 3).

The following 'challenge studies' were designed to further examine methionine metabolism in pancreatitis. They are based on the work of Horowitz who utilised a 'loading approach' to examine the transsulphuration pathway in cirrhotics (Horowitz 1981). That paper reported, an elevated plasma concentration and retarded clearance of methionine in their patients with cirrhosis. By utilising the 'challenge studies' described in that paper, we planned to study patients with pancreatic disease, in the expectation that it would accentuate and allow

identification of the site of any absolute, covert or relative block causing disruption of the methionine transsulphuration pathway. We planned to compare plasma methionine concentration at entry (baseline) and then peak plasma concentrations, plasma half-life, metabolic clearance rate and the area under the curve (AUC) over an 8 hour period following an oral methionine load, seeking differences between individuals with a history of pancreatitis and healthy controls.

We anticipated that patients with pancreatitis might have a greater area under the curve due to the combination of higher baseline levels, prolonged plasma half-life and slower metabolic clearance rates secondary to 'blockade(s)' in the transsulphuration pathway, as found in patients with liver disease. It was also expected that as a result of differences in methionine pharmacokinetics, that the peak concentration would be higher in subjects with a history of pancreatic disease.

GSH (γ-L-glutamyl-L-cysteinylglycine), is the major intracellular non-protein thiol, acting in the intracellular compartment. It is present in all mammalian tissue, including the pancreas, where it acts as a cytoprotective agent against noxious stimuli capable of initiating pancreatitis. Through both conjugation and redox reactions GSH functions as an antioxidant. Pancreatic GSH levels are reduced in caerulein-induced pancreatitis, and its role in maintaining pancreatic integrity has been discussed in earlier chapters. Depletion of GSH can initiate pancreatitis (Dabrowski 1990, Lüthen 1994) and augmentation of its synthesis protect against the disease in experimental models (Neuschwander-Tetri 1992, Wang 1996).

The transsulphuration pathway supplies the cell with sufficient cysteine to keep up with the requirements of γ-glutamylcysteine synthetase (Neuschwander-Tetri 1992). We thus planned to document blood glutathione concentrations before and periodically after oral NAC or methionine loading, to determine whether baseline levels were suppressed in individuals with a history of pancreatitis and whether oral sulphur amino acid supplementation, produces any detectable elevation in GSH concentrations.

The rate limiting step of transsulphuration is the conversion of homocysteine to cystathionine by the enzyme cystathionine β-synthetase, Figure 1.3. (Finkelstein 1978). By loading an individual with a metabolite that feeds into the pathway above or below this irreversible step, it is possible to investigate either the whole or just the distal-most aspects of the pathway. Thus, we reasoned that methionine loading would examine the entire pathway. But, loading with N-acetylcysteine (NAC), because it is converted to cysteine and thus feeds into the transsulphuration pathway below cystathionine, Figure 1.3., we would accentuate only defects in the later metabolic steps of transsulphuration. By comparing differences in the plasma methionine, cystine, GSH and taurine (another end product of transsulphuration) concentrations following methionine and NAC loads it was anticipated that the site of any vulnerable step(s) would be revealed.

The lack of benefit observed in the NAC / SAMe trial described in Chapter 2, may be explained in part by coexistent low selenium and ascorbate levels, neither of which were corrected by the interventional regime tested. Selenium, an antioxidant in its own right, is an essential cofactor for the enzyme glutathione peroxidase. This catalyses reduction of potentially highly toxic peroxide moieties thus:

$$ROOH + 2GSH \xrightarrow{\textit{glutathione peroxidase}} ROH + GSSG + H_2O$$

Ascorbate is the principal aqueous phase antioxidant. One of its functions is in the reduction of oxidised glutathione by both enzymic and non-enzymic interactions between 2 redox shuttles (Winkler 1992). Indeed a degree of redundancy has been observed between ascorbate and glutathione (Märtensson 1993, Treacy 1996). Thus, failing to address a lack of either of these micronutrients, attempts to normalise glutathione metabolism, especially in the face of unfettered oxidative stress, might have been predicted to fail.

Section 1.2.1. reviewed the phenomenon of oxidative stress. A number of transsulphuration pathway enzymic steps (labelled A - G in Figure 1.3.) are potentially susceptible to oxidative stress (Nunn 1982, Davies 1986). The

benefit of micronutrient antioxidant supplementation in patients with recurrent acute (non-gallstone) or chronic pancreatitis, in both the short and intermediate term (Uden 1990, Bilton 1994, Sharer 1995) and in the long term (McCloy 1998), support the Manchester hypothesis of the aetiology of pancreatitis: that oxidative stress from heightened free radical exposure, due either to direct exposure or increased production, in the presence of insufficient or reduced antioxidant defences, results in the disturbance to exocytosis - 'pancreastasis' that heralds an attack of pancreatitis (Braganza 1991, 1996).

Dietary intake of antioxidants is less in patients with chronic pancreatitis than controls (Rose 1986). Thus we anticipated low levels of micronutrient antioxidants and vitamins in the challenge study pancreatitis patients at the time of entry into the challenge studies. By performing challenge studies, by loading with oral doses of methionine and NAC in individuals with RAP and CP, before and after ten weeks of oral micronutrient antioxidant supplementation therapy (AOT), the protocol would examine the transsulphuration pathway in the presence and absence of oxidative stress. The oral supplement used, Bioantox, (*PharmNord UK*) comprised selenium, methionine and vitamins A, C and E, taken on a daily basis. We predicted that any 'blockade' identified in the transsulphuration pathway with methionine and / or NAC loading prior to AOT would be partially or wholly 'normalised' by the supplementation regime that had proven to be effective in clinical practice (Uden 1990a, Bilton 1994, McCloy 1998).

4.1.1. Further protocol development.

The automated amino acid analyser acquired and methodology developed for methionine quantitation in these studies, allowed for the simultaneous estimation of the other plasma amino acids. Of these, the following were selected to focused upon:

1. ***Taurine*** - as an end product of transsulphuration, plasma levels would be expected to fall, as is seen with GSH, if transsulphuration is interrupted.

Taurine is a particularly interesting amino acid for since these studies were first designed, the potent antioxidant properties of taurine have been highlighted. Taurine is recognised to be protective against ethanol-induced hepatic steatosis and lipid peroxidation (Kerai 1998), thioacetamide-induced hepatic cirrhosis (Balkan 2001) and streptozotocin-induce diabetes in rats (Chang 2000). A recent publication has reported that taurine supplementation is also able to protect rats from cerulein-induced pancreatitis (Ahn 2001).

2. ***Glycine*** - with cysteine and glutamate are constituents of the tripeptide, glutathione. If a rate limiting block in the transsulphuration pathway is present, or be accentuated by sulphur amino acid loading in patients with pancreatic disease, then cysteine availability might limit glutathione synthesis. As a result plasma glycine concentration might be expected to rise. Furthermore, glycine is intimately connected with one-carbon and folic acid metabolism. Glycine links with folic acid metabolism in 2 ways:

a) $\text{Glycine} + \text{THF} + \text{NAD}^+ \longrightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{NADH} + \text{H}^+ + N^5,N^{10}\text{-methylene- THF}$

b) $\text{Glycine} + N^5,N^{10}\text{-methylene-THF} \longrightarrow \text{THF} + \text{serine}$

Figure 3.1.1. shows the complex relationship of glycine to the recycling of homocysteine to methionine. In this reaction 5-CH_3-THF, ultimately derived from the N^5,N^{10}-methylene-THF product of reactions a) above, donates its methyl group to homocysteine. If plasma methionine is elevated in pancreatitics as in cirrhosis, then recycling requirements might be reduced, 5-CH_3-FH_4 utilisation would fall and hence levels might rise. This could be reflected in rising glycine levels, as would the negative inhibition from rising SAH and 5-methyltetrahydrofolate concentrations to the reaction in which glycine is methylated by SAMe to sarcosine.

3. ***Serine*** - is combined with homocysteine by cystathionine synthetase to form cystathionine. A block in transsulphuration proximal to homocysteine would reduce the rate of this metabolic step and a rise in serine might be anticipated. Serine and glycine are closely linked through folate metabolism, Figure 3.1.1. and thus should be measured simultaneously.

4. ***Valine*** - a branched chain amino acid that is catabolised to propionyl-CoA, is also an intermediate of methionine degradation. The penultimate enzymic step that converts both valine and methionine to succinyl-CoA, a tricarboxylic acid cycle intermediate, is methylmalonyl-CoA-mutase. Thus, elevated methionine levels could saturate this degradation pathway, reducing valine breakdown, resulting in rising plasma concentrations. Methylmalonyl-CoA-mutase, like methionine synthetase is a vitamin B_{12} dependent enzyme. Vitamin B_{12} deficiency would inhibit both enzymes, whereas oxidative inactivation of B_{12} has only been reported of the latter enzyme (Sharer 1983).

5. ***Cystine*** - the disulphide dimer of cysteine which is in equilibrium with the free reduced form and mixed disulphide with the cysteinyl residues of albumin or other plasma proteins. Chawla *et. al.* 1984 reported the plasma levels of these compounds in patients with cirrhosis and compared them to those found in normal individuals, Table 4.1.1. over page. Corresponding values in patients with pancreatic disease are unknown.

	Normal subjects (n)	Cirrhotic patients (n)	Students t-test
Methionine	31.9 +/- 2.6 (14)	59.0 +/- 20.6 (10)	NS
Cysteine	11.2 +/- 0.9 (14)	6.9 +/- 1.1 (10)	0.005
½ Cystine	79.4 +/- 7.6 (14)	88.6 +/- 22.4 (10)	NS
Protein-bound cysteine	150.6 +/- 5.3 (14)	153.5 +/- 16.9 (10)	NS
Total plasma cyst(e)ine	241.3 +/- 10.2 (14)	254.9 +/- 14.8 (10)	NS
Cyst(e)ine thiol to disulphide ratio	0.051 (14)	0.030 (10)	0.01
Glutathione	4.5 +/- 0.4 (30)	2.0 < 1 (6)	0.01
Total thiol	439 +/- 27.4 (10)	355 +/- 42.5 (4)	NS
Albumin	701.7 +/- 22.6 (6)	401.8 +/- 61.1 (4)	0.001

Table 4.1.1. Fasting plasma thiol concentrations μM +/- SEM in normal individuals and cirrhotic patients while on a normal diet. NS = not significantly different. *After Chawla et. al. 1984*

6. ***Urinary inorganic sulphate*** - In man on an omnivorous diet, most of the sulphur derived from amino acids not required for protein synthesis is degraded to inorganic sulphate and excreted in the urine. Some of the inorganic sulphate is conjugated with other compounds (e.g. phenols) to form ester sulphates. Other sulphur containing compounds are found in urine and are referred to as the non-sulphate sulphur fraction (e.g. methionine, NAC and mercaptolactate). where;

   non- sulphate sulphur = total sulphur - total sulphate (inorganic sulphate + ester sulphate).

   Urinary inorganic sulphate reflects total intake of sulphur-containing amino acids and excretion is recognised to fall during starvation (Märtensson 1982) or during

diets low or deficient in sulphur (Sabry 1965). Its metabolic synthesis is depicted in Figure 4.1.1. The activity of cysteine dioxygenase, the rate limiting enzyme in inorganic sulphate and taurine formation, is reported to fall in sulphur amino acid depleted rats (Märtensson 1982). This might lead to a rise in the production of mercaptopyruvate and mercaptolactate, components of the non-sulphate sulphur pool.

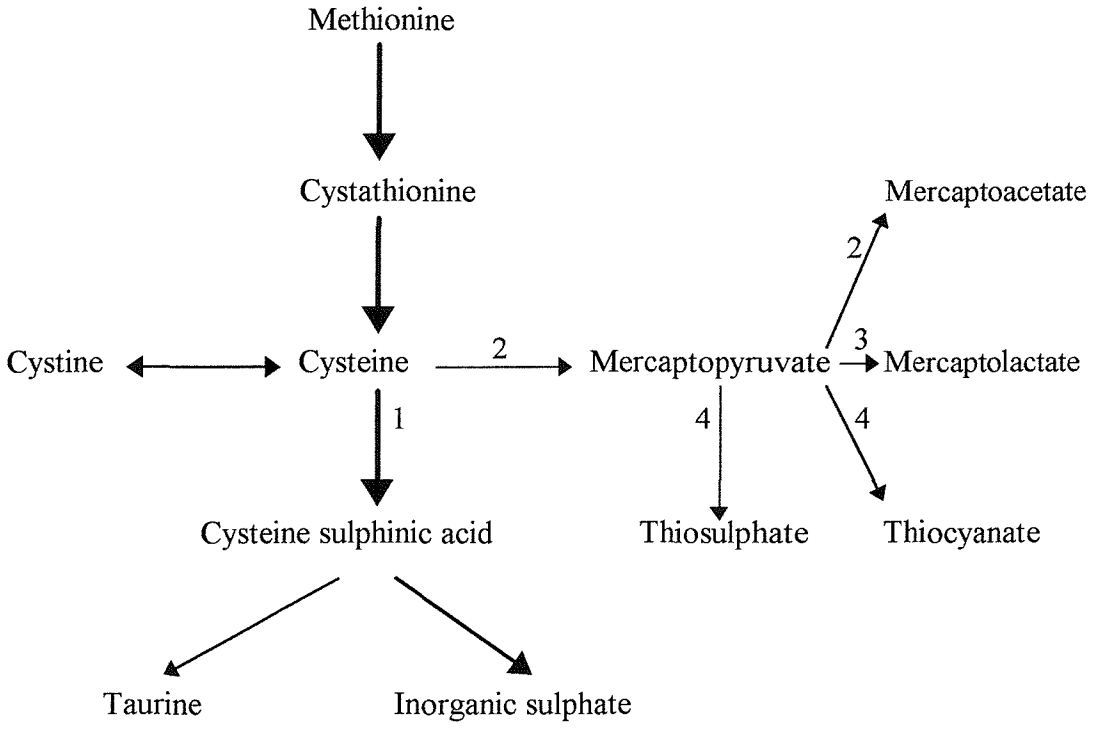


Figure 4.1.1. Metabolic degradation of sulphur amino acids (*after Märtensson 1982*). (1 - cysteine dioxygenase: 2 - cysteine aminotransferase: 3 - lactate dioxygenase: 4 - mercaptopyruvate sulphurtransferase).

Urinary inorganic sulphate concentrations were measured for comparison in our subjects as a way of assessing pre-supplementation sulphur intake, integrity of the production pathways and as an indirect measure of compliance with AOT.

7. ***Micronutrient antioxidants and markers of free radical activity:*** To assess susceptibility to oxidative stress, fasting blood samples were taken either at the

time of recruitment or on the morning of the first challenge study (prior to oral dosing), for analysis of the micronutrient antioxidant vitamins. The following antioxidants were measured, serum selenium, β-carotene, α-tocopherol and retinol, and plasma vitamin C. As a marker of free radical activity the molar ratio of 9,11 / 9,12 linoleic acid, a free radical mediated isomerisation, was quantitated. The methodology for these analyses are described in Appendix A.

In order to confirm concordance and to document the effect of oral supplementation therapy, a representative number of subjects had further samples analysed after 5 and 10 weeks therapy.

4.1.2. Summary of hypotheses under investigation

a) Is fasting plasma methionine higher in patients with a history of pancreatitis than normal subjects, i.e. similar to that reported in cirrhosis, and if so, is this corrected or exacerbated by AOT containing methionine?

b) if fasting plasma methionine is higher, can the step(s) at which flux through the transsulphuration pathway is disrupted causing the difference between the groups be identified?

c) if a) proves negative, could a covert anomaly in flux through the transsulphuration pathway be accentuated and identified by stressing the pathway by oral loading, in a similar manner to that used to identify heterozygotes for homocysteinuria (Sardharwalla 1974)? Identification of the site of any insufficient step in transsulphuration in pancreatitics would be aided by loading the pathway with substances that augment transsulphuration pathway intermediates before (methionine) and after (NAC) the rate-limiting enzymic step, i.e. that catalysed by cystathionine β-synthase.

d) that the plasma concentrations of other amino acids, intimately linked to methionine metabolism, are abnormal in patients with pancreatitis.

e) that oxidative stress is present and can be detected in subjects with a history of pancreatitis, and

f) that by correcting oxidative stress with oral micronutrient supplementation, any identifiable 'blockade' in methionine metabolism can be eliminated or reduced.

4.2.0. Study subjects

Control subjects were outwardly healthy volunteers drawn from employees of Manchester Royal Infirmary or their friends and family. Exclusion criteria included:

i) Age less than 18 years of age.

ii) Pregnancy.

iii) Previous personal history of pancreatic disease.

iv) Family history of pancreatic disease.

v) Current or recent antioxidant or vitamin supplements.

vi) Inability to give informed consent.

Volunteer control subjects were entered into the study following a clinical history, physical examination and an abdominal ultra-sound scan to confirmed normal pancreatic morphology.

Patients with a history of pancreatitis were recruited from consecutive patients referred to the Manchester Royal Infirmary Pancreato-Biliary Service, between June 1992 and September 1995. Exclusion criteria included:

i) Age less than 18 years of age.

ii) Pregnancy.

iii) Current or recent antioxidant or vitamin supplements.

iv) Opiate analgesic dependency.

v) Inability to give informed consent.

4.2.1. Definition of pancreatitis

The diagnosis in patients referred to The Pancreato-Biliary Service with pancreatitis was confirmed on clinical criteria and if necessary further specific investigations.

Recurrent acute pancreatitis: was accepted as the diagnosis on the basis of two or more previously documented attacks of acute pancreatitis - defined as an admission amylase at least 3 times the upper limit of normal, and/or evidence of acute pancreatitis at laparotomy or radiological (ultra-sound / CT) scanning, in the presence of a compatible clinical history. Patients potentially suitable and

consenting to entry were reviewed in the Pancreatic clinic 6 weeks or longer after resolution of their most recent acute episode. Entry at that point was dependent upon them being asymptomatic, with no persistent biochemical (elevated serum amylase) or radiological (ultra-sound scan) evidence of pancreatic disease.

Chronic pancreatitis was accepted as a diagnosis on the basis of definitive histology or radiological evidence of pancreatic calcification or advanced-change ductal disease on ERCP. Patients possessing an inflammatory pancreatic mass and / or a pseudocyst, on ultrasound or CT scanning, more than 6 weeks after an attack of acute pancreatitis, were also considered suitable for inclusion.

Challenge Study Protocol.

4.3.0. Pre-challenge assessment

Suitable individuals were seen in the Pancreato-Biliary out-patient clinic the week prior to the first study. The following baseline observations were recorded:-

i) *Haematology* - FBC, ESR, serum B_{12}, ferritin, serum and RBC folate.

ii) *Biochemistry* - U & E's, creatinine, LFT's, calcium, caeruloplasmin, iron and total iron binding capacity (TIBC), α1-antitrypsin, fasting lipid profile and glucose.

iii) ***Antioxidant and markers of free radical activity*** - Serum selenium, α-tocopherol, β-carotene and retinol; plasma vitamin C, whole blood and plasma GSH and 9-11 / 9-12 linoleic acid ratio.

4.3.1. Dietary assessment and preparation

An equilibrating 40g protein diet was implemented 72 hours before each challenge study began. The diet was tailored to ensure it contained sufficient calories to meet basal metabolic requirements. A pair of scales was loaned to facilitate accurate home preparation using food that would suit each individuals usual dietary preference.

Patients were instructed on protein content of food and provided with printed protein exchange example sheets (Appendix B). Food diary record sheets were kept for the three days prior to each challenge and 'compliance' with the protocol assessed by one of the hospital's dietitians. Study subjects were given the telephone number, on which they were free to contact a dietitian at any stage if they needed to discuss their intake during the three day 'lead-in' period or were unsure of the protocol to follow.

Printed instructions were given of the day and time on which to start the three day lead-in diet, menu memo sheets, the method for collecting a 24 hour urine collection and the time from when to be nil by mouth for the challenge study.

4.3.2. Thumb-nail plan of study protocol

Subject recruitment — *day minus 7*

Baseline haematology, biochemistry and oxidative stress markers — *day minus 7*

Start 40g / day protein diet — *day minus 3*

Start 1st 24 hr urine collection — *day minus 1*

Phase 1 (NAC 1 **OR** METH 1) — *day 0*

Complete 12 - 24 hr urine collection — *day 1*

Resume normal diet — *day 1*

Restart 40g / day protein diet — *day 11*

Start 1st 24 hr urine collection — *day 13*

Phase 2 (METH 1 **OR** NAC 1) — *day 14*

complete 12 - 24 hr urine collection — *day 15*

Pancreatitic subjects start (*Bio-antox* i qid), resume normal diet. — *day 15*

Outpatient assessment +/- oxidative stress markers — *day 50*

Restart 40G / day protein diet — *day 82*

Start 1st 24 hr urine collection — *day 84*

Phase 3 (NAC 2 **OR** METH 2) — *day 85*

complete 12 - 24 hr urine collection — *day 86*

Resume normal diet — *day 86*

Restart 40g / day protein diet — *day 96*

Start 1st 24 hr urine collection — *day 98*

Phase 4 (METH 2 **OR** NAC 2) — *day 99*

complete 12 - 24 hr urine collection — *day 100* ***End***

4.3.3.1. Challenge studies: Phase 1

Day minus 3: Commence 40g protein / day diet. Drink fluid *ad libitum*.

Day minus 2: Continue 40g protein / day diet.

Day minus 1: Continue 40g protein / day diet and at 08.30 start 24 hour urine collection.

Starve from midnight.

Day 0: Admit to Ward 26; Royal Infirmary, Manchester at 08.00. Weigh to nearest kilogram.

08.30: Complete 24 hour urine collection and commence 1st 6 hour collection.

08.40: Insert intravenous cannula (14G) in ante-cubital fossa and take base-line blood sample (12ml) for sulphur amino-acids, and if not taken at the pre-assessment clinic, blood for antioxidants, markers of free radical activity and glutathione levels (30ml).

09.00: Administer oral challenge dose:-

i) L- Methionine (*Pharma Nord* UK) 50 mg/kg + sucrose 0.675 g/kg **or**

ii) N-Acetylcysteine (Fabrol - *Zyma Healthcare UK*) 50 mg/kg in 120ml carbohydrate-free juice.

PharmaNord UK. kindly manufactured and donated the *L-methionine* in 100 and 500mg tablets specially for these studies.

Subjects received an oral load of L-Methionine and N-Acetylcysteine a fortnight apart. The order in which the challenge compounds in the first phase were administered, was determined at random, by the toss of a coin. In subjects with

pancreatic disease the oral loading studies were repeated after 10 weeks micronutrient antioxidant supplementation. The order in which the two compounds were given in this second phase was the same as in the first, Table 4.3.1.

09.30: 12ml blood for sulphur amino-acids and glutathione. Subjects were then allowed protein-free breakfast. This comprised tea or coffee with cream / water to whiten in place of milk, toast (protein-free bread) + thick margarine + jam, 100 ml fruit juice and water as required.

10.00: 12ml blood for sulphur amino-acids and glutathione.

10.30: 12ml blood for sulphur amino-acids and glutathione.

12.00: 12ml blood for sulphur amino-acids and glutathione. Followed by lunch prepared in the specialist renal kitchen within the hospital. The day's diet was designed to contain 40G protein in total, with calories coming from carbohydrate and fat (i.e. protein sparing effect). This involved the addition of proprietary products like Maxijul (carbohydrate source) + / - Duocal (fat / carbohydrate emulsion).

13.30: 12ml blood for sulphur amino-acids and glutathione.

14.30: Complete 1st and start 2nd six hour urine collections.

15.00: 12ml blood for sulphur amino-acids and glutathione.

18.00: 12ml blood for sulphur amino-acids and glutathione. Followed by evening meal, prepared in the specialist renal kitchen within the hospital, designed for the 40G protein per day diet.

20.30: Complete 2nd six hour and start 12 - 24 hour urine collections.

21.30: Snack and drink to complete 40G protein diet.

Day 1: 08.*30*: Complete 12 - 24 hour urine collection and allow normal breakfast as desired. Discharged home with printed instructions on date on which to start the protein-restricted diet again in preparation for the second phase.

4.3.3.2. Phase 2

Day 11 - 13 as for days minus 3 - minus 1 in phase 1.

Day 14: As for day 0 in the first phase, except at 08.00 no requirement for repeat base-line blood for antioxidants and markers of free radical activity. 12ml blood taken for sulphur amino-acids and glutathione.

09.00: Administer the alternative challenge compound to the first phase. Thus if L-methionine was selected at random in phase one, N-Acetylcysteine is given in phase two and *vice versa*.

Day 15: As for day 1, but subjects with RAP and CP were supplied with sufficient Bioantox (*PharmaNord UK*) to allow a dose of one tablet six hourly (*q.i.d.*) for the next 10 weeks. These individuals were booked an out-patient follow-up for their 'week five check' and set a date for phase three and four. The normal controls were not given antioxidant supplementation and were not entered into phase 3 or 4.

4.3.3.3. Week 5 check (day 50)

Clinical records were updated with frequency and severity of any relapses, consultations with primary or secondary care physician and analgesic requirements. A specific record was made as to the acceptability of the Bioantox tablets and the occurrence of adverse reactions. Compliance was assessed by verbal discussion, a tablet count and fasting blood concentrations of plasma vitamin C, serum selenium

and whenever possible serum β-Carotene, α-tocopherol and retinol. Blood samples were also taken to estimate whole blood and plasma glutathione.

4.3.3.4. Phase 3

Days 82 - 84: As for days minus 3 to minus 1 of phase 1, except take last Bioantox tablet at 22.00 on day 84.

Day 85: As for day 0, including the administration of the same challenge compound at dose of 50 mg/kg at 09.00.

Day 86: Restart antioxidant tablets at08.30 at end of 12 - 24 hour urine collection.

4.3.3.5. Phase 4

Days 96 - 98: As for days 11 - 13 in phase 2, except take last Bioantox tablet at 22.00 on day 3.

Day 99: As for phase 2, including the administration of the same challenge compound at dose of 50 mg/kg at 09.00.

Day 100: Complete 12 - 24 hour urine collection. Recommend continuation of Bioantox and arrange routine clinical follow-up through Pancreato-Biliary clinic as indicated by clinical criteria.

4.3.3.6. Oral Antioxidant Supplement Preparation

The *Bioantox* tablets were produced by *PharmaNord UK* and dispensed by the pharmacy department of Manchester Royal Infirmary. Each *Bioantox* tablet contained the following within an outer coating that contained 1mg riboflavin as colouring:

i) Selenium 75μg ii) β-Carotene 7.2mg iii) Vitamin C 150mg

iv) Vitamin E 47mg v) L-methionine 400mg.

TABLE 4.3.1.

Order in which compounds were administered in the challenge studies

Controls initials	1st challenge compound
HBS	NAC
PFN	Methionine
EGY	Methionine
MHN	NAC
AJN	Methionine
GKY	NAC
NST	Methionine
OSN	Methionine

RAP subjects initials	1st challenge compound
RMcE	NAC
EGY	Methionine
DHD	Methionine
PHS	Methionine
CKN	NAC
EMcE	Methionine
JMK	Methionine
SWS	Methionine
PFH	NAC

CP subjects initials	1st Challenge compound
JCN	Methionine
JGH	NAC
NHN	Methionine
LJS	Methionine
GMN	Methionine
POE	NAC
EPY	NAC

4.3.4. Challenge study specimen collection

Physiological specimens were processed using by the following methodology:

4.3.4.1. Blood:

a) ***Serum***: Samples to quantitate serum selenium, β-carotene, α-tocopherol, 9,11 - 9,12 - linoleic acid molar ratio and amylase concentrations required 20ml venous blood. It was collected into plain tubes and a clot allowed to form and retract whilst on ice. The samples were spun down at 1500g (3000 rpm) for 10 minutes at 4°C, serum separated off and stored as ~ 10 x 0.8 ml aliquots at -70°C until analysed.

b) ***Plasma***: 11ml blood was collected into heparinised tubes and spun down at 700g (2000 rpm) at 4°C for 15 minutes. The separated plasma was deproteinised by a precipitant with vortexing to ensure though mixing and left on ice for 10 minutes before further vortexing and being centrifuged at 1500g for 20 minutes at 4°C. The precipitants used were as follows:

i) 2 volumes plasma + 1 volume 12% sulphosalicylic acid (SSA) stored as x5 200μl samples in Eppendorfs for analysis of plasma methionine, taurine, glycine and other amino acids, plus a single 1.2 ml stored for future SAMe and cysteine quantitation.

ii) 2 volumes plasma + 2 volumes 10% trichloroacetic acid (TCA) stored as x3 200μl samples in Eppendorfs for free homocysteine and the remainder as a single aliquot for vitamin C estimation.

All deproteinised plasma samples were stored at - 70°C until analysis.

c) Whole blood: 5 ml blood collected into EDTA tube and processed immediately, at the bedside for plasma and whole blood glutathione, see Appendix A. The remainder was sent for routine haematological analysis as a full blood count (FBC), to be utilised in the calculation of GSH concentration.

4.3.4.2. Urine:

The volume of the 24 hour urine collection prior to each challenge study was recorded and the following fractions stored after preparation in the following manner.

i) 3 x 7 ml and 2 x 1 ml aliquots frozen at - 20°C for urinary sulphate excretion and creatinine, respectively.

ii) 6 x 1 ml aliquots frozen at - 70°C for urinary sulphur amino acids.

iii) 20 ml urine + 500µl 10% w/v ascorbic acid for urinary folate assay.

Urine produced over the 24 hours following administration of challenge substance was collected in 2 x 6 hour and 1 x 12 hour collections. The volume of each fraction was recorded and samples prepared and stored as above.

Unfortunately time limitations permitted only inorganic sulphate : creatinine and folate analysis from these samples to be performed.

4.4.0. Ethics

The studies were passed by the Ethics Committee of The Central Manchester Healthcare Trust. Patients and healthy controls were provided with an information sheet that outlined the background to the studies as well as the study protocol. Written informed consent was obtained after they had been given time to read and consider the request and had their questions answered. The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki.

4.5.0. Calculations

4.5.1. Area under the curve (AUC)

AUC calculations of graphs of methionine, other amino acids and glutathione were calculated on the equation:

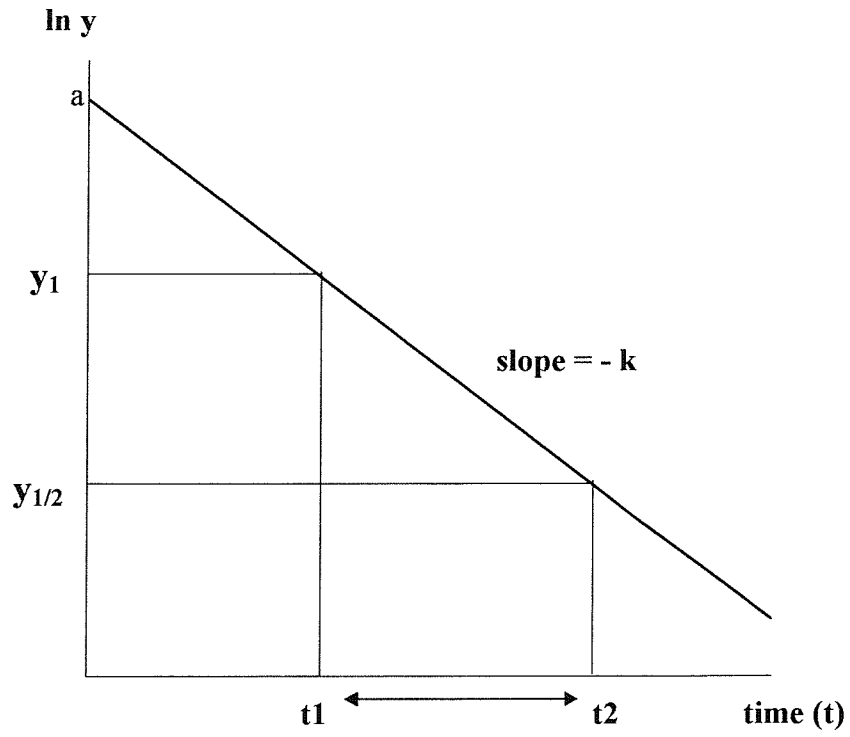
$AUC = [(X_0 + X_1)/2]0.5 + [(X_1 + X_2)/2]0.5 + [(X_2 + X_3)/2]0.5 + [(X_3 + X_4)/2]1.5 + [(X_4 + X_5)/2]1.5 + [(X_5 + X_6)/2]1.5 + [(X_6 + X_7)/2]3$

where X_0 is the y value at time zero, X_1 is the y value at time 30 minutes and so on and so forth.

4.5.2. Plasma half life ($t_{½}$)

The **half life** ($t_{½}$) was calculated using the formula:

$t_{½} = \ln 2 / k$ which is derived thus:



$\ln y = a - kt$

$\ln y_1 = a - kt_1$

$\ln y_1 = a - kt_2$

$$t_2 - t_1 = [(a - \ln y_1/2)/k] - [(a - \ln y_1)/k]$$

$$= 1/k (\ln y_1 - \ln y_1/2)$$

$$= 1/k (\ln y_{1/} y_{1/2}) \qquad = 1/k \ln(2)$$

$$= \ln(2)/\text{slope}$$

In order to utilise the maximum number of points to calculate the slope, t½ and the most accurate intercept point (a), different t_1 values were taken depending on the time that the maximum concentration was recorded in each group in each challenge study and t_2 was selected to include the exponential fall in concentration following the peak. These values ranged from 180 - 540 minutes post dose for plasma methionine concentration following a methionine load, to 30 - 360 minutes post dose for plasma cystine concentrations following a NAC challenge in the control group.

The t½ calculation assumed exponential decay. The value for methionine concentration at 180 - 540 minute were used in the following equation in Excel 95:-

t ½ = ln(2) / [- slope {ln[methionine]180, ln[methionine]270, ln[methionine]360) ln[methionine]540},{180, 270, 360, 540}]

4.5.3. Volume of distribution (V_d)

The Volume of distribution (V_d) is the ratio of the administered dose to the concentration at time zero. The latter of these values is established by extrapolating the graph backwards to the y-intercept. The concentration in µmol/l is the anti-ln of this intercept value - calculated thus :

$$2.718281\text{^ y-intercept or } (e^y).$$

The volume of distribution (V_d) was calculated by back extrapolation of the methionine curves assuming exponential decay, as depicted in figure 4.5.1.

4.5.4. Metabolic clearance rate (MCR)

Metabolic clearance rate (MCR) is the product of volume of distribution ($V_{d)}$ and elimination rate constant (K_e) where:

$$Ke = \ln(2) / t½$$

$K_e = 0.693/t½$ thus:

$$MCR = [\ (0.693 / t½)\ V_d\ ]\ 1000 \text{ ml/min}$$

4.6.0. Statistics

The *Excel for Windows 95* computer package was utilised for spreadsheet data management, statistical analysis and plotting of graphs. Comparisons between the pharmacokinetic parameters derived from the profiles of the amino acids analyses in the three subject groups were made utilising the students t-test. The 'paired' t-test was used for comparisons within groups e.g. subjects with recurrent acute pancreatitis before and after micronutrient antioxidant supplementation and 'unpaired' [assuming unequal variances (heteroscedastic)] for analyses between the groups. The Bonferroni correction procedure was used in calculations where multiple-comparisons were made. For example, our hypothesis was that *baseline* methionine levels would be higher in patients with pancreatitis than controls. Thus to compare methionine levels at the seven other time points of a challenge study (30, 60, 90, 180, 270, 360 and 540 minutes), in the three groups (controls, RAP, CP) a p-value of < 0.0024 (based on a level of $p < 0.05$ for a single comparison) was required to reach statistical significance. Non-parametric statistics utilised the Wilcoxon Rank Sum Test with the null hypothesis being disproved for $p < 0.05$.

4.7.0. Plasma amino acid concentration: principles

Plasma amino acid concentrations represent a balance between their intestinal absorption, biosynthesis, protein synthesis, renal tubular reabsorption and catabolism. There is usually an equilibrium between enhanced amino acid metabolism and protein synthesis in the anabolic, fed state and degradation of proteins with consumption and release of amino acids into the free amino acids 'pool' when an animal is catabolic or fasted. Man is able to synthesise only ten of the twenty amino acids, which means those that cannot be produced by intermediate metabolism are required as 'essential' in the diet. Methionine is one of these essential amino acids and the effect of dietary insufficiency on the pancreas has been discussed in Chapter 1. Methionine metabolism through the transsulphuration pathway involves enzymes that are dependent upon co-enzymes for their activity (Figures 3.1.1., 4.1.1.). Deficiency or inactivation of these cofactors, vitamins or trace elements may contribute to a metabolic blockade of the pathway, causing abnormal levels of intermediate metabolites.

4.7.1. Amino Acid Analysis: Methodology.

Plasma amino acid analysis was performed using a *Pharmacia* LKB 4151 *Alpha plus Amino Acid Analyser*. This automated analyser utilises a 20cm x 4.6mm column, containing an 8-μm cation-exchange resin that separates amino acids by ion exchange. After a period of trial and experimentation, step-wise elution using five lithium-citrate buffers was selected, under the following programming conditions to achieve optimal separation and resolution of the peaks of interest:-

i) Buffer 1 (pH 2.80, 0.2M) was pumped for 7 minutes, followed by
ii) Buffer 2 (pH 3.00, 0.3M) for 28 minutes,
iii) Buffer 3 (pH 3.02, 0.6M) for 27.5 minutes,
iv) Buffer 4 (pH 3.45, 1.0M) for 19.5 minutes, and
v) Buffer 5 (pH 3.55, 1.65M) for 39 minutes.

The column was regenerated with 0.3M lithium hydroxide for 6 minutes followed by equilibration in buffer 1 for 32 minutes. During this cycle the column was initially heated to 34°C for 46 minutes and then raised to 65°C for 54 minutes, then 75°C for 35 minutes before returning the column to 34°C. The complete cycle took 159 minutes.

The flow rate was 25 ml/hour for each of the buffers. The amino acids were detected with ninhydrin reagent pumped at 20 ml/hour through a reaction coil maintained at 135°C. The optical density (O.D.) range / gain was set at 1.0 for both the 440-nm and 570-nm absorbance. Peak assignment was established by the sequential spiking of a deproteinised plasma sample with solutions of known amino acids (Sigma Chemicals). Norleucine was chosen as the internal standard as it is not naturally occurring and elutes close to the amino acid of major interest i.e. methionine. Once the identity of the peaks of interest had been established, the chromatograms were recorded by an integrator in series, rather than the dual channel chart recorder, with the 570 nm output serving as the permanent record, Figures 4.7.1. and 2.

Plasma samples deproteinised with sulphosalicylic acid (SSA) (separation methodology discussed in section 4.3.4.1.), were stored in 200µl aliquots at -70° C. For analysis, the contents of the Eppendorfs were thawed at room temperature and 5µl internal standard of 20.5mM norleucine, made up in loading buffer (buffer 1: pH 2.80, 0.2M) added to each vial. The vials were then vortexed and spun at 2,800g for 10 minutes in a microcentrifuge.

Sample loading cartridges with capacity of 200µl, were rinsed with methanol, washed through and primed with the loading buffer. A test sample volume of 60µl plasma containing the internal standard was added to the cartridge and 10µl loading buffer added after the sample. Ten sample cartridges were prepared daily and the amino acid analyser run continuously when possible to reduce variation in retention times and separation. A stock amino acid standard solution (Sigma Chemical Company, product no. A-9906) with norleucine as internal standard was prepared

(thus 200µl standard plus 200µl loading buffer and 10µl of norleucine 20.5 mM, stored at 4°C).

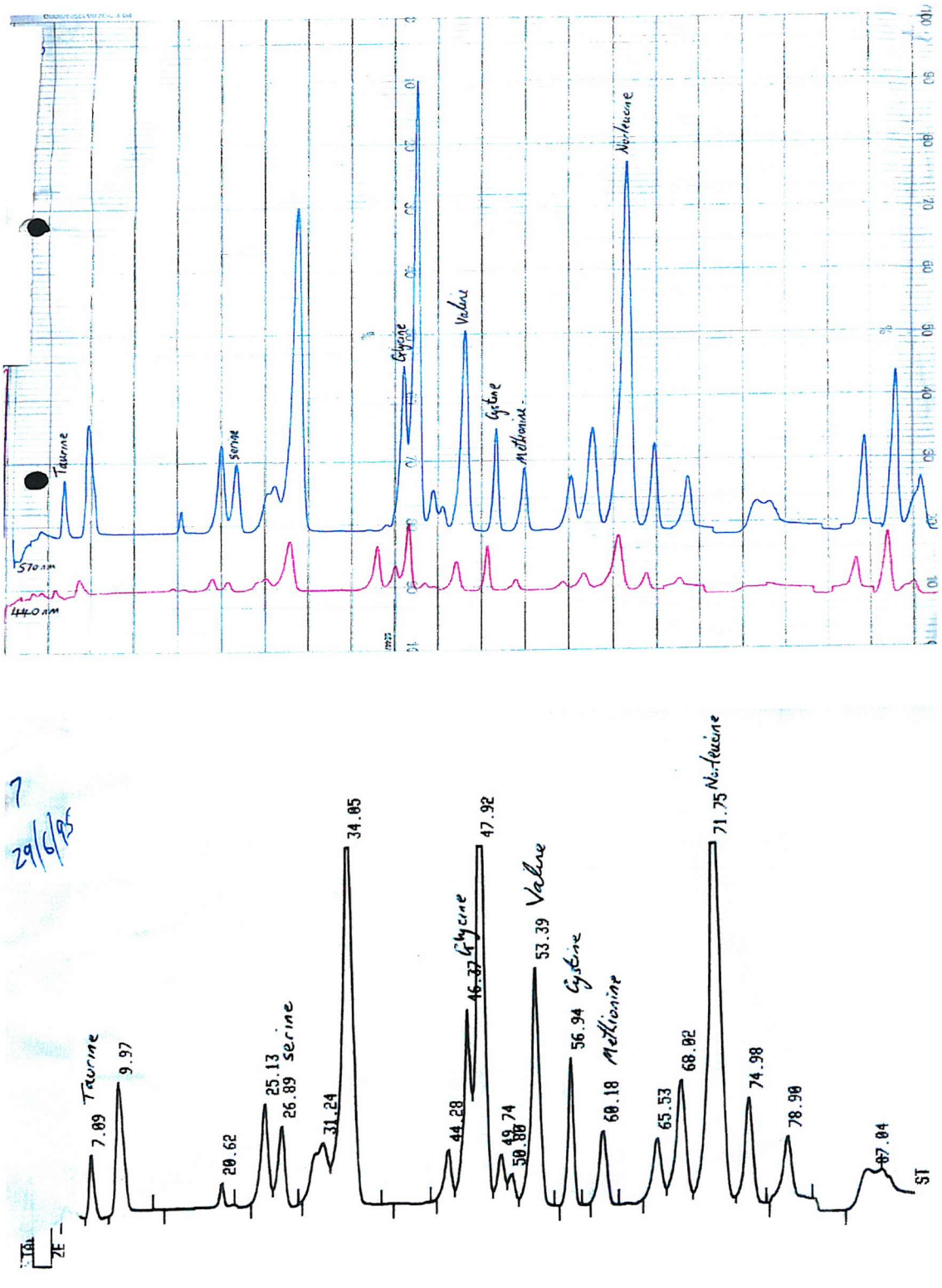


Figure 4.7.1. Chromatograms showing the analysis of the free plasma amino acids in 60ml of deproteinised human plasma, with 5ml norleucine (20.5 mM) internal standard. Both dual channel pen recorder and integrator in series traces shown with peaks of interest identified.

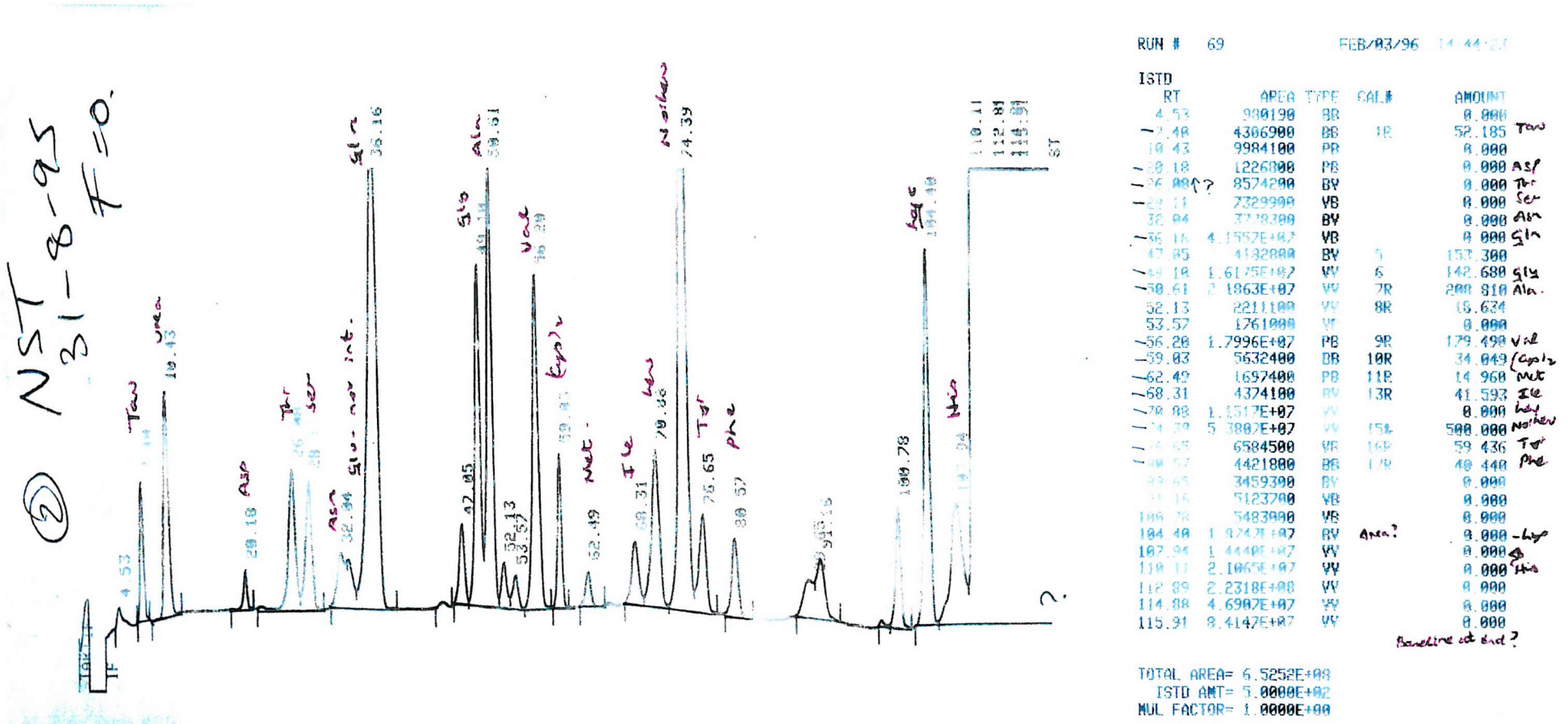
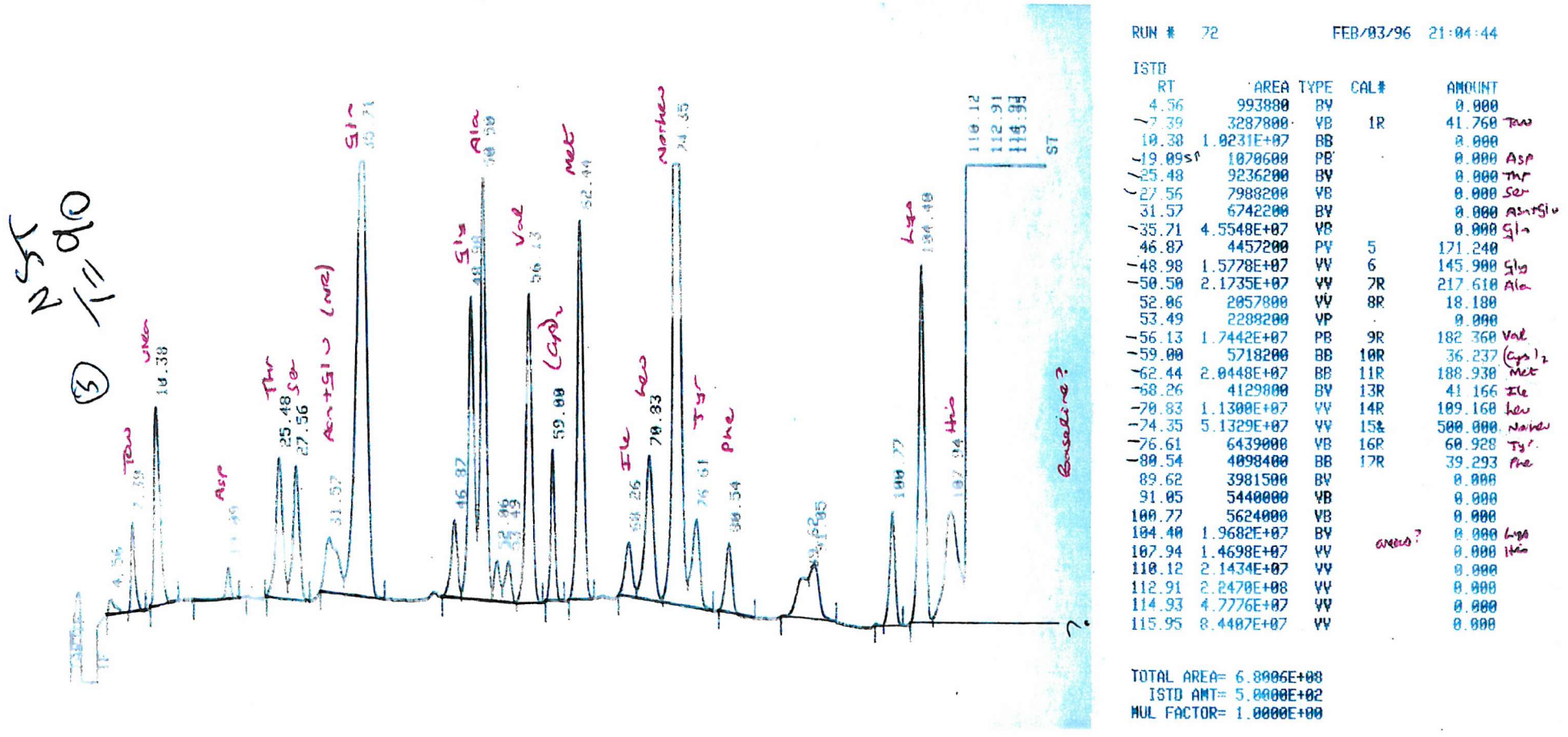


Figure 4.7.2.a. Examples of integrator record of deproteinised plasma amino acid analysis in control subject (NST) at baseline.



RUN # 72 FEB/03/96 21:04:44

ISTD RT	AREA	TYPE	CAL#	AMOUNT	
4.56	993880	BV		0.000	
7.39	3287800	VB	1R	41.760	Tau
10.38	1.0231E+07	BB		0.000	
19.09	1070600	PB		0.000	Asp
25.48	9236200	BV		0.000	Thr
27.56	7988200	VB		0.000	Ser
31.57	6742200	BV		0.000	Asn+Gln
35.71	4.5548E+07	VB		0.000	Gln
46.87	4457200	PV	5	171.240	
48.98	1.5778E+07	VV	6	145.900	Gly
50.50	2.1735E+07	VV	7R	217.610	Ala
52.06	2057800	VV	8R	18.180	
53.49	2289200	VP		0.000	
56.13	1.7442E+07	PB	9R	182.360	Val
59.00	5718200	BB	10R	36.237	(Cys)2
62.44	2.0448E+07	BB	11R	188.930	Met
68.26	4129800	BV	13R	41.166	Ile
70.83	1.1300E+07	VV	14R	109.160	Leu
74.35	5.1329E+07	VV	15R	500.000	Norleu
76.61	6439000	VB	16R	60.928	Tyr
80.54	4098400	BB	17R	39.293	Phe
89.62	3981500	BV		0.000	
91.05	5440000	VB		0.000	
100.77	5624000	VB		0.000	
104.40	1.9682E+07	BV		0.000	Lys
107.94	1.4698E+07	VV		0.000	His
110.12	2.1434E+07	VV		0.000	
112.91	2.2470E+08	VV		0.000	
114.93	4.7776E+07	VV		0.000	
115.95	8.4407E+07	VV		0.000	

TOTAL AREA= 6.8006E+08
ISTD AMT= 5.0000E+02
MUL FACTOR= 1.0000E+00

Figure 4.7.2.b. Integrator record of deproteinised plasma amino acid analysis in control subject (NST) 90 minutes after oral Methionine load, demonstrating the rise observed in methionine concentration.

4.7.2. Quality control of amino acid analyses

Three approaches to quality assurance were designed into the analyses.

1. ***Physiological standard samples*** (Sigma) were run in order to calibrate the system and calculate plasma levels. However, as this standard was run at least every tenth cartridge, by comparing the standard results at either end of an analytical run, a degree of quality assurance was obtained. This allowed small variations in retention time to be tracked and separation of peaks for acceptable resolution to be monitored. Plasma amino acid concentrations in the experimental samples were generally calculated using the mean of the *Sigma* standard sample performed immediately before and after the sample batch run, corrected for internal standard. If the mean area ratio between the two physiological standards for any amino acid of interest, varied by greater than 10%, then the standard most representative of the chromatograms surrounding it was used for calculation. Analysis of experimental samples was deemed acceptable if resolution of the peaks of particular interest was inadequate or the baseline proved so unstable that both integrator calculation of the peak area or extrapolation to allow the peak to be cut out and weighed were unreliable.

2. ***Sulphur amino acid recovery experiment***. A single unit of pooled plasma was obtained from the North West Regional Blood Transfusion Service. This was deproteinised with 12% SSA as were the experimental samples (2 volumes plasma : 1 SSA). Three batches of 12 separate plasma volumes were deproteinised in this way from the pooled sample. Batch 1 was deproteinised without prior spiking (P1-12). In order to estimate the efficiency of recovery batch 2 was 'spiked' with a 'low' concentration of methionine, cysteine and homocysteine (M1-12), while the final aliquots were 'spiked' with high concentrations (H1-12), prior to deproteinising. Table 4.7.1. reports the additions of sulphur amino acids made to the pooled plasma. Recovery of added amino acids was assessed following chromatographic analysis.

	Concentration of solution added (10μl) to 3 ml plasma		
Sulphur amino acid	**P1-12**	**M1-12**	**H1-12**
Methionine	nil	18mM	0.174M
Cysteine	nil	9mM	27mM
Homocysteine	nil	75mM	0.675M

Table 4.7.1. Preparation of spiked pooled plasma control specimens

3. In addition, these pooled plasma samples, stored at -70° C, labelled P1-12; M1-12; H1-12 were analysed thus:
    i) Duplicates of the same sample at each concentration e.g. P5, M7 and H2 on consecutive sample cartridges to assess intra-assay variability.
    ii) Samples prepared separately but run consecutively e.g. P1/P2, M3/M4 and H9/H10 assesses intra-assay variability, but also takes into account imprecision in the deproteinisation stage.
    iii) Repeated analysis of the pooled plasma samples, periodically over the duration of the experimental sample analysis, to confirm stability in storage e.g. P3 repeated six months apart.

After May 1999 the *Pharmacia* LKB 4151 analyser was decommissioned. This was whilst the data of the amino acid analyses were being examined. At this time it became apparent that a number of the chromatograms had achieved inadequate separation to permit sufficiently accurate integration of peaks for acceptable quantitation. A total of 45 samples were therefore re-analysed by the Department of Chemical Pathology at Southampton General Hospital, on their fully automated, *Pharmacia Biotech* amino acid analyser (*Biochrom 20*). This offered the opportunity of an additional, but originally unplanned quality assurance mechanism. Six of the pooled plasma samples (P4-6 and M4-6) were analysed with the repeated experimental samples. The Department of Chemical Pathology is a CPA accredited

laboratory and participates in the international **'Erndim'** quantitative external quality assessment scheme (*www.erndimqa.nl*).

4.7.3. Calculations

Physiological standards: The Sigma standard (500μM of each amino acid, 200μl) was diluted with 200μl loading buffer, then10ml Norleucine (20.5mM) added.

Norleucine in physiological standard solution calculation:

$$[\text{Norleucine}] = 10/(10+200+200) \times 20.5\text{mM} = 500\mu\text{M}$$

$$\text{other [amino acids]} = 200/(10+200+200) \times 500\mu\text{M} = 244\mu\text{M}$$

:- if amino acids give same sensitivity at 570nm as does norleucine, then following ninhydrin reaction, expect

$$\text{mean (area ratio to norleucine)} \times 1000 = 244/500 \times 1000 = 488$$

Cystine gives approximately double this figure as 1 molecule contains two amino residues and thus reacts with twice the ninhydrin.

Experimental samples:

Physiological standards are diluted x 2 prior to addition of internal standard.

Experimental samples are diluted x 3/2 prior to addition of internal standard.

$$\text{Thus [sample amino acid } \mu\text{M]} = \frac{\text{sample amino acid area}}{\text{sample norleu area}} \times \frac{500 \times 1000 \times 1/2 \times 3/2}{\text{mean (area ratio to norleu)}}$$

$$\text{Thus [sample amino acid } \mu\text{M]}^{\star} = \frac{\text{sample amino acid area}}{\text{sample norleu area}} \times \frac{375000}{\text{mean (area ratio to norleu)}}$$

Peak areas were entered manually onto a Microsoft Excel spreadsheet file and concentration calculated using the formula* above.

4.7.4. Quality Assurance

At physiological concentrations, plasma homocysteine and cysteine (reduced forms) are either so low that their peaks are too small to quantify, or the conditions of the chromatogram fail to achieve separation. After oral loading with NAC or methionine, neither homocysteine and cysteine peaks became identifiable. Quantifying recovery from the spiking experiments for either of these amino acids was not therefore performed. The cystine peak changed after cysteine spiking as a consequence of auto-oxidation.

Assessing the results obtained from duplicate estimation of the physiological standards, the following percentage difference in concentration were found, table 4.2. These figures are calculated from values that exclude any where the percentage difference exceeded the 10% cut-off level (these standards were not used in calculation of experimental concentrations from peak areas). From 113 matched standard pairs, taurine was the amino acid which most frequently yielded unacceptable differences in peak areas. The reason for this observation is that this is the first amino acid eluted off the column and in retrospect a longer equilibration period would have been beneficial when the machine was being set up from a standstill. Any error introduced because of this will not be transferred to experimental sample analysis, because a physiological standard analysis was routinely performed first whenever the analyser was started up and the serine peaks, eluted at 29 minutes, do not show evidence of any inconsistency due to separation conditions.

Percentage difference between all paired standards	**Taurine** (n=88)	**Serine** (n=106)	**Glycine** (n=104)	**Valine** (n=107)	**Cystine** (n=110)	**Methionine** (n=109)
Mean	3.74	2.7	2.68	2.25	1.97	2.08
Median	3.50	1.90	2.10	1.85	1.30	1.15

Table 4.7.2. Percentage difference between peaks obtained from the physiological standard samples corrected for internal standard.

Analysis of the pooled plasma control samples yielded the reproducibility figures reported in tables 4.7.3 - 4.7.5. Of the 12 basic (non-spiked) pooled samples, analysis was repeated 28 times. Samples spiked with a 'medium' amino acid concentrations ('M') were quantitated 21 and those with 'high' concentration ('H') 17 times. The mean (s.d.) methionine concentration derived from the individual samples was: P1-12 – 30.1 (2.4), M1-12 - 90.0 (7.0) and H - 586.1 (30.1).

Thus in the 'medium' group:

Added [methionine] $= (90.1 - 30.1) +/- \sqrt{(7.0)^2 + (2.4)^2}$

$= 59.7 +/- 7.4$ μmol/l

:- recovery 100 +/- 12%

In the 'high' group:

Added [methionine] $= (586.1 - 30.1) +/- \sqrt{(30.1)^2 + (2.4)^2}$

$= 556.0 +/- 28.6$ μmol/l

:- recovery 100 +/- 5%

thus in both cases recovery was quantitative. Note that as expected the levels of taurine, serine, glycine and valine were unaffected.

The most any single preparation was analysed was four in the case of P5. The mean methionine value in these estimations of P5 = 30.3μmol/L (s.d. 3.2).

Plasma concentrations μmol/l	Taurine	Serine	Glycine	Valine	Cystine	Methionine
mean of individual results (n=28)	50.2	87.5	208.5	256.9	94.5	30.1
standard deviation	6.1	8.0	20.8	15.4	7.0	2.4
SEM	1.2	1.5	3.9	2.9	1.3	0.4

Table 4.7.3. Reproducibility data for non-spiked pooled plasma samples

Plasma concentrations μmol/l	Taurine	Serine	Glycine	Valine	Cystine	Methionine
mean of individual results (n=21)	49.8	86.8	204.9	252.3	134.4	90.0
standard deviation	7.2	7.4	16.2	19.4	22.8	7.0
SEM	1.6	1.6	3.5	4.2	5.0	1.7

Table 4.7.4. Reproducibility data for 'M' spiked pooled plasma samples

Plasma concentrations μmol/l	Taurine	Serine	Glycine	Valine	Cystine	Methionine
mean of individual results (n=17)	50.5	86.8	203.6	257.5	341.0	586.1
standard deviation	11.5	5.9	10.8	14.7	94.0	30.1
SEM	2.8	1.4	2.6	3.6	22.8	7.3

Table 4.7.5. Reproducibility data for 'H' spiked pooled plasma samples

When the same samples (P4-6) were analysed 6 months apart, and then 5 years later, no significant difference on long term storage, was observed for the stability

of amino acids, except cystine. Apart from cystine, plasma concentrations differed by Cystine values were lower after prolonged storage and thus values obtained after 5 year storage were rejected. Cystine values were lower after 5 year prolonged storage and thus these values were not included in subsequent calculations.

Plasma conc. μmol/l	Taurine	Serine	Glycine	Valine	Cystine	Methionine
P4 *(n=3 in 1995)*	51	86	201	248	93	30
P4 *(n=1 in 2000)*	45	86	203	257	49	27
P5 *(n= 4 in 1995/6)*	49	91	215	263	97	31
P5 *(n = 1 in 2000)*	45	86	203	257	49	28
P6 *(n=2 in 1995)*	60	102	262	290	103	33
P6 *(n = 1 in 2000)*	44	86	203	258	49	28

Table 4.7.6. Inter-assay variability in plasma control, analysed at Manchester in 1995/6 and after 5 years storage at - 70°C repeated in 2000 at Southampton.

4.7.5. Peak weighing: When peak area was incorrectly assessed by the integrator or when visual inspection revealed that base line drift or inappropriate designation of the beginning and / or end of the peak by the integrator was wrong, the peak area was quantified by weight. The procedure for this was:

i) photocopy chromatogram x 200% enlargement

ii) carefully draw in baseline for peak of interest and for a peak of known area. Norleucine peak was chosen whenever possible for this process.

iii) The 'unknown' and 'known' peaks were carefully cut out and weighed on a Cahn micro-balance to microgram precision.

Then: Area unknown peak = area known peak x $\frac{\text{mass unknown peak}}{\text{mass known peak}}$

When the area of unknown peak has been established the concentration is then calculated in the usual manner.

Results

4.8.0. Demographics of study subjects

Eight outwardly healthy controls and 16 patients with pancreatic disease were studied. Nine of the patients had a diagnosis of recurrent acute and seven had chronic pancreatitis by our diagnostic entry criteria. The demographic details including alcohol and smoking history and occupational history appear in tables 4.8.0.1. - 4.8.0.3. No difference in age at entry or body weight was noted between the controls and the patients with pancreatitis. The control group had fewer current smokers compared to those with pancreatic disease. The patient group were also heavier previous drinkers of alcohol. Of the patients studied, one with RAP (PFH) and one with CP (NHN) withdraw after phase 2 of the challenge studies (both because they no longer wished to take part, rather than for any clinical reason). The results of Phase 3 and 4 were therefore calculated on the basis of the remaining 14 patients.

4.8.1. Routine blood studies

Baseline haematology was normal in all subjects, tables 4.8.1.1. - 4.8.1.3. and liver function tests were within the laboratory reference range in all but two patients. In one of these (PFH) with recurrent acute pancreatitis, the elevated alkaline phosphatase was interpreted as secondary to Paget's disease on the basis that the ALP was predominately the bone isoenzyme. Secondly, a 32 year old man (POE) with chronic calcific pancreatitis had abnormal alkaline phosphatase, bilirubin and hepatic transaminases. He had an inflammatory mass in his pancreatic head, resulting in early extrahepatic biliary obstruction (as evidenced by a moderately dilated common bile duct of 8mm but without intra-hepatic biliary dilatation on ultra-sound scanning), mild cholestasis and a possible alcoholic hepatitis. Histological confirmation was not considered indicated on clinical criteria. His serum amylase was also the only one to be elevated at the time of entry to the study, at 541 iu/L (NR 44 - 200). His serum pancreatic isoamylase was 93.9μg/l (NR 6.8 - 29.8) at the time of entry to the challenge studies.

Challenge Study Controls **Table 4.8.0.1.**

Initials	Gender	Age at entry	Weight (kg)	Smoking (pack years)[a]	Alcohol (units/week) [b]	Occupational hydrocarbon exposure[c]
HBS	M	49	87	Nil	10	++
PFN	F	61	74	Nil	12	nil
EGY	F	34	57	Nil	15	nil
MHN	M	50	95	Nil	40	+
AJN	M	37	76	Nil	Nil	nil
GKY	M	52	77	10 (Ex)	Nil	nil
NST	M	20	77	0.5	40	nil
OSN	F	60	66	21 (Ex)	3	nil
Mean	*5M : 3F*	*45*	*76*			

Legend to tables 4.8.0.1. - 4.8.0.3:

[a] Smoking 20 cigarettes a day for 1 year is equivalent to 1 pack year.

[b] Alcohol intake of controls was a reported average over previous 5 years. None of the controls had previously consumed alcohol at a level higher than currently reported. The alcohol intake of individuals with pancreatitis is reported as maximum level sustained for at least a twelve month period as most had either stopped or dramatically reduced their intake after developing pancreatitis.

[c] Occupational exposure to inhaled hydrocarbons is a recognised factor in the aetiology of pancreatitis (Braganza 1986a, Sandilands 1989, McNamee 1994). + represents low and ++ moderate and high levels of exposure.

Challenge Study Recurrent Acute Pancreatitics **Table 4.8.0.2.**

Initials	Gender	Age	Weight (kg)	Clinical history	Aetiology	Smoking (pack years) [a]	Max. alcohol (units/week) [b]	Occupational hydro-carbon exposure [c]
RMcE	M	39	100	AP 1992 & 93	alcohol	31 (Ex)	Ex - 200	nil
EGY	F	65	70	AP 1984 & 93	gallstone	24 (Ex)	2	nil
DHD	M	54	80	AP 1987 & 93	alcohol	20	Ex - 140	+
PHS	M	42	80	AP 1974 - 94	idiopathic	5 (Ex)	30	nil
CKN	F	25	58	AP 1993 x 2 (pre- & post cholecystectomy)	gallstone	nil	nil	+
EMcE	F	32	89	AP 1987& 1992 with transient (pseudo)cyst	gallstone	8.5	21	+
JMK	M	32	62	AP 1990, 92 & 93	alcohol	14	80	nil
SWS	M	31	78	AP 1989, 90 & 93	alcohol	9	60	++
PFH	F	61	69	AP 1981 & 93	gallstone	17 (Ex)	nil	nil
Mean	*5M : 4F*	*42*	*76*					

Challenge Study Chronic Pancreatitics **Table 4.8.0.3.**

Initials	Gender	Age	Weight (kg)	Pancreatic morphology	Smoking (pack years) [a]	Max. alcohol (units/week) [b]	Occupational hydro-carbon exposure [c]
JCN	M	44	70	Chronic calcific pancreatitis with small pancreatic cysts	28	Ex - 120	++
JGH	M	49	65	Chronic calcific pancreatitis and portal vein thrombosis	30	Ex - 300	+
NHN	M	24	68	Non-calcific advanced-change pancreatitis on ERCP	10	Nil	+
LJS	F	23	81	Calcific advanced-change pancreatitis on ERCP	1.4	25	nil
GMN	F	83	56	5.5cm mass HoP with dilated PD and atrophy of body/tail	Nil	10	nil
POE	M	32	74	Chronic calcific pancreatitis with intermittent jaundice	15	Ex- 120	+
EPY	M	46	74	Moderate change on ERCP and 5cm pseudocyst in body	20	Ex - 55	nil
Mean	*5M:2F*	*43*	*70*				

Table 4.8.1.1.

Controls - baseline laboratory measurements.

Initials	Baseline FBC			Baseline LFTs				
	Hb g/dl	WBC $x10^9$/l	Platelets $x10^9$/l	Bilirubin µmol/l	Albumin g/l	ALP iu/l	ALT iu/l	AST iu/l
HBS	13.9	5.8	214	13	41	138	21	15
PFN	11.7	5.2	253	8	42	114	15	15
EGY	12.2	6.9	256	6	41	128	7	12
MHN	12.8	4.3	260	11	40	121	30	29
AJN	15.8	5.8	271	33	46	200	31	20
GKY	13.3	8.4	233	12	39	101	16	19
NST	15.0	11.0	222	7	48	202	21	24
OSN	13.4	4.7	224	16	42	164	14	19

Hb = haemoglobin g/dl, WBC = white blood cell $x10^9$/l, ALP = alkaline phosphatase iu/l, ALT = alanine transaminase iu/l, AST = Aspartate transaminase iu/l.

[AJN's isolated hyperbilirubinaemia was accepted as Gilbert's syndrome].

Table 4.8.1.2.

Recurrent acute pancreatitics - baseline laboratory measurements.

	Baseline FBC			Baseline LFT's				
Initials	**Hb g/dl**	**WBC $x10^9$/l**	**Platelets $x10^9$/l**	**Bilirubin µmol/l**	**Albumin g/l**	**ALP iu/l**	**ALT iu/l**	**AST iu/l**
RMcE	14.9	8.5	277	8	46	208	42	28
EGY	12.6	5.1	224	11	43	219	27	30
DHD	14.1	9.6	266	8	46	178	27	18
PHS	13.1	3.9	246	10	45	115	26	18
CKN	12.1	7.6	211	8	42	100	23	19
EMcE	13.1	8.8	254	8	41	279	16	15
JMK	15.8	6.4	310	8	48	132	34	23
SWS	14.6	7.3	206	14	46	181	21	22
PFH	13.6	8.6	278	5	43	500	21	18

Table 4.8.1.3.

Chronic pancreatitics - baseline laboratory measurements.

Initials	Baseline FBC			Baseline LFT's				
	Hb g/dl	WBC $x10^9$/l	Platelets $x10^9$/l	Bilirubin mmol/l	Albumin g/l	ALP U/k	ALT U/l	AST U/l
JCN	14.6	11.5	410	10	43	301	21	15
JGH	13.0	7.1	236	7	39	166	29	23
NHN	15.3	9.0	247	9	45	150	29	19
LJS	12.5	4.3	301	6	47	219	29	20
GMN	10.9	5.0	193	4	40	303	22	19
POE	13.5	9.1	256	31	46	434	356	233
EPY	13.9	5.3	221	7	45	186	26	21

4.8.2. Additional biochemical analyses

Table 4.8.2.1. shows that there was no difference between the three groups with respect to serum iron. TIBC was higher in subjects diagnosed as having chronic pancreatitis than in controls. These results differ from a previous study that compared normal subjects with patients having chronic calcific pancreatitis at Manchester, UK and Johannesburg, RSA. In that report, serum iron and TIBC levels were similar in all four groups (Segal 1996). However, in line with that study, we confirmed that the combined group of patients with a history of pancreatitis (RAP + CP) have higher serum caeruloplasmin concentrations than controls. Furthermore, the finding of elevated triglycerides (Durrington 1986, Toskes 1994) and calcium (Mithöfer 1995), both aetiological agents for the development of RAP, was in line with expectation. Blood α1-antitrypsin was higher in both groups of patients with pancreatitis than in controls.

	Cholesterol mmol/l	TG mmol/l	Calcium mmol/l	Iron µmol/l	TIBC µmol/l	α1-AT g/l	Caeruloplasmin g/l
Controls							
HBS	5.1	0.7	2.17			1.28	0.28
PFN	4.9	1.1	2.30	18.3	51	0.64	0.33
EGY	3.6	0.9	2.23	10.8	48	1.74	0.55
MHN	4.4	0.8	2.15			1.33	0.25
AJN	5.1	1.2	2.27			1.44	0.46
GKY	5.5	1.3	2.11	19.9	38	1.17	0.3
NST	4.7	2.0	2.37	22.6	50	1.23	0.31
OSN	6.8	1.3	2.23	20.2	44	1.26	0.35
RAP							
RMcE	6.5	1.6	2.40	10.3	52	1.45	0.43
EGY	5.5	2.6	2.40	14.3	46	1.58	0.51
DHD	8.7	8.5	2.35	11	47	1.55	0.47
PHS	7.4	2.0	2.19	16.3	55	1.68	0.26
CKN	3.3	0.7	2.35			1.83	0.61
EMcE	5.8	3.0	2.30	15.2	54	1.97	0.57
JMK	5.8	2.1	2.54	20.1	66	1.6	0.4
SWS	5.6	3.1	2.29	12.3	46		0.64
PFH	4.8	1.9	2.33	15	50	1.45	0.56
CP							
JCN	5.8	2.6	2.33	19.6	54		
JGH	3.4	1.3	2.22	4.6	55		0.36
NHN	4.7	1.0	2.20				0.28
LJS	4.9	0.8	2.27	14.3		1.73	0.45
GMN	4.4	2.0	2.39	14.2	62	2.55	0.45
POE	8	6.4	2.39	34.8	62	1.32	0.49
EPY	5.5	0.5	2.32	14.9	52	1.81	0.5
Normal range							
low	-	0.8	2.15	7	45	1	0.25
high	5.2	1.8	2.65	29	70	2	0.63

	Cholesterol	TG	Calcium	Iron	TIBC	α1-AT	Caeruloplasmin
c v rap	ns	0.02	0.05	ns	ns	0.005	0.05
c v cp	ns	ns	ns	ns	0.010	0.05	ns
c v allp	0.05	0.05	0.02	ns	0.05	0.005	0.05
rap v cp	ns	ns	ns	ns	ns	ns	ns

c - controls
rap - recurrent acute pancreatitis
cp - chronic pancreatitis
allpanc - combined rap and cp patients

Additional Baseline Biochemistry values

Table 4.8.2.1. lower table - probability by Wilcoxon Rank Sum Test

4.8.3.1. Plasma Methionine

Baseline plasma methionine was similar in the three subject groups (controls, recurrent acute and chronic pancreatitis). Challenging subjects with NAC or methionine did not alter the subsequent baseline fasting plasma methionine concentration, a fortnight later, when the second phase of the challenge studies was performed.

		Phase 1	Phase 2	Phase 2
		Baseline	after methionine 1st	after NAC 1st
Control	*mean*	23.9	24.6	22.3
	sd	4.4	5.2	4.0
RAP	*mean*	21.7	22.8	25.3
	sd	3.0	3.2	4.0
CP	*mean*	21.3	19.0	22.3
	sd	2.9	3.9	9.1

Table 4.8.3.1.1. Baseline plasma methionine (μmol/l) mean and s.d. for groups in phase 1 and phase 2 divided by first challenge compound received.

When individuals were separated by gender, there appeared to be a difference in fasting plasma methionine, between male and female patients with recurrent acute pancreatitis, after AOT. This difference, (mean +/- s.d.) males 25.2 μmoles/l +/- 4.2 {n=5}; females 17.7 μmoles/l +/- 1.2 {n=3}, $p < 0.014$ was observed in the methionine but not the NAC challenge study, Table 4.8.3.1.2. However, the Bonferroni correction factor of 6 comparisons (3 groups each of 2 genders) suggests that this difference is not relevant for a $p < 0.008$ would be required to reach significance. Furthermore, no difference was seen in the control or CP group, or in the RAP group prior to AOT and is therefore most unlikely to be a true difference. It is therefore assumed that there is no difference between genders and results are subsequently analysed as mixed gender groups.

Following an oral load of methionine, plasma levels rose to a peak between 90 and 180 minutes after ingestion, of up to twenty fold over baseline values. The

individual plasma methionine profiles in each of the 24 subjects challenged are shown in Appendix C.

Figures 4.8.3.1.1. - 3. show the collated individual profiles of plasma methionine in the three subject groups i.e. controls, patients with RAP or CP, in phases 1 and 2 of the challenge studies, i.e. before AOT. Figures 4.8.3.1.4. - 5. show the individual profiles of subjects with pancreatitis in phases 3 and 4, i.e. after AOT. Figure 4.8.3.1.6. - 7. show mean plasma methionine profile for the three subject groups, following an oral methionine challenge, before and after AOT. These results are tabulated, Table 4.8.3.1.3. No discernible difference was found between the subject groups in either the fasting baseline concentration (time zero) or in the peak methionine level (180 minutes) following an oral methionine load, before AOT. In the profiles following AOT, plasma methionine at 180 minutes, for patients with CP was less than those with RAP ($p = 0.02$). A significant difference also appeared to be present between the control and chronic pancreatitis subjects at the 30 minutes post loading time point, both before and after AOT, table 4.8.3.1.3. However, these fail to reach significance after correction for the six comparisons made at each time-point. Furthermore, no difference persisted at the 60 minute or subsequent time points, suggesting that the difference was a reflection of delayed absorption, rather than in the rate of methionine metabolism.

The mean plasma methionine profiles during the challenge studies, for each of the three subject groups are shown in figures 4.8.3.1.8 - 10 The **A**rea **U**nder the **C**urve (AUC) of these methionine profiles, show no discernible difference between the three subject groups within any single challenge study. Micronutrient antioxidant supplementation did not result in the anticipated reduction of AUC in the methionine profile in subjects with either RAP or CP, Table 4.8.3.1.3 and Figure 4.8.3.1.11.

Figure 4.8.3.1.12. collates and compares plasma methionine profiles of all the pancreatic patients when their results are combined into a single study group in the four challenge studies, against the control subjects. Figure 4.8.3.1.13. shows the

methionine profiles, following the NAC challenges seen in Figure 4.8.3.1.12, on a larger scale. Again, control and patient groups were inseparable.

The mean plasma half life (t½) of methionine following an oral load of 50mg/kg was the same in the three subject groups, (range 257 - 293 minutes), Table 4.8.3.1.4. The individual plasma methionine half-lives in each phase of the challenge studies are plotted with their group means in Figure 4.8.3.1.14, where RAP1 is the recurrent acute pancreatitis patients before AOT and CP2 represents the patients with chronic pancreatitis after AOT. No significant difference was demonstrated between t½ in the three groups, nor did AOT supplementation result in any change, Table 4.8.3.1.4.

Volume of distribution (V_d) and metabolic clearance rate (MCR) of methionine were similar in the three subject groups, as found in the group formed by combining all 16 patients with pancreatic disease (referred to as *allpanc*), in Table 4.8.3.1.5. As with the other pharmacokinetic parameters, ten weeks micronutrient antioxidant supplementation did not alter either V_d or MCR for these groups.

Plasma methionine concentration fell over the first 3 hours following oral NAC, but subsequently returned towards baseline levels.. Figures 4.8.3.1.15. - 21. show the individual and group plasma methionine profiles following oral loading of NAC, in the NAC 1 (before AOT) and NAC 2 (after AOT) phases of the challenge studies. The mean and standard deviations tabulated in Table 4.8.3.1.6. shows that plasma methionine is lower 270 minutes after an oral NAC load in patients with RAP, compared to healthy controls after ten weeks AOT. No difference was observed between the AUC of plasma methionine following oral NAC in the 3 subject groups, irrespective of antioxidant status.

Statistical comparisons confirms a progressive fall in plasma methionine concentration for the first 4 hours following oral NAC, with recovery to pre-dose levels thereafter, Table 4.8.3.1.7. These values are highly significant, but no difference was identified between the three groups examined before AOT or in the

patient groups after AOT. Thus it is acceptable to combining all individual values (control, RAP and CP) at each time point. This increased the level of significance of the difference between the baseline level and subsequent time points, with the greatest depression in plasma methionine concentration being observed 180 minutes post dose, p = 2E-11, Table 4.8.3.1.7.

Summary of plasma methionine results

- Fasting plasma methionine levels are normal in patients with quiescent pancreatitis.
- Oral methionine results in peak plasma levels 90 to 180 minutes later.
- During the first three hours after oral NAC plasma methionine falls progressively, before returning to pre-dose level over the subsequent three hour period.
- 10 weeks AOT did not alter fasting methionine concentrations or the peak value observed following an oral load.
- There was a trend for plasma methionine levels of patients with CP to be lower than those of controls, thirty minutes after a methionine load, irrespective of antioxidant status. No other differences at individual times were observed.
- Plasma methionine t½ following an oral load of methionine, is similar in normal subjects and individuals with a history of recurrent acute or chronic pancreatitis.
- AOT had no significant effect on the t½, V_d and MCR of plasma methionine following an oral load.
- AOT does not change the mean AUC of plasma methionine, after an oral challenge of methionine or NAC, in patients with a diagnosis of pancreatitis.

		control NAC	control METH	RAP NAC	RAP METH	CP NAC	CP METH	[1]before AOT [2]after AOT	t tests NAC	t tests METH
Before AOT	**means**	24.0	23.6	22.2	23.1	20.0	21.8	c v rap1	**0.326**	**0.815**
Both genders	**sd**	4.2	4.8	2.7	3.9	3.3	6.3	c v cp1	**0.058**	**0.578**
After AOT	**means**			25.5	22.3	23.2	23.7	rap1 v cp1	**0.174**	**0.672**
	sd			4.8	5.0	8.2	2.2	rap2 v cp2	**0.552**	**0.521**
Before AOT	**means**	25.6	24.2	23.0	24.6	21.5	22.2	c v rap1	**0.295**	**0.887**
Male	**sd**	4.0	4.8	3.3	3.8	1.9	7.0	c v cp1	**0.089**	**0.614**
After AOT	**means**			27.4	25.2	21.3	24.0	rap1 v cp1	**0.426**	**0.336**
	sd			4.3	4.2	8.6	3.0	rap2 v cp2	**0.349**	**0.669**
								cp1 v cp2	**0.501**	**0.814**
								rap1 v rap2	**0.224**	**0.612**
Before AOT	**means**	21.3	22.7	21.3	21.3	16.0	20.0	c v rap1	**0.972**	**0.736**
Female	**sd**	3.5	5.9	1.7	3.7	2.8	n/a	c v cp1	**0.168**	**n/a**
After AOT	**means**			22.3	17.7	20.0	22.5	rap1 v cp1	**0.191**	**n/a**
	sd			4.2	1.2	4.2	0.7	rap2 v cp2	**0.600**	**0.010**
								cp1 v cp2	**0.570**	
								rap1 v rap2	**0.678**	**0.212**
		c(m) v c(f)		rap(m) v rap(f)	rap(m) v rap(f)	cp(m) vs cp(f)	cp(m) vs cp(f)			
t tests **Before AOT**		**0.176**	**0.723**	**0.345**	**0.224**	**0.173**	**n/a**			
After AOT				**0.168**	**0.014**	**0.834**	**0.268**			

Table 4.8.3.1.2. Baseline Fasting Plasma Methionine (μmol/l) in single- and mixed-gender groups (mean + s.d.)

Figure 4.8.3.1.1. Plasma methionine profiles in controls after methionine challenge before AOT

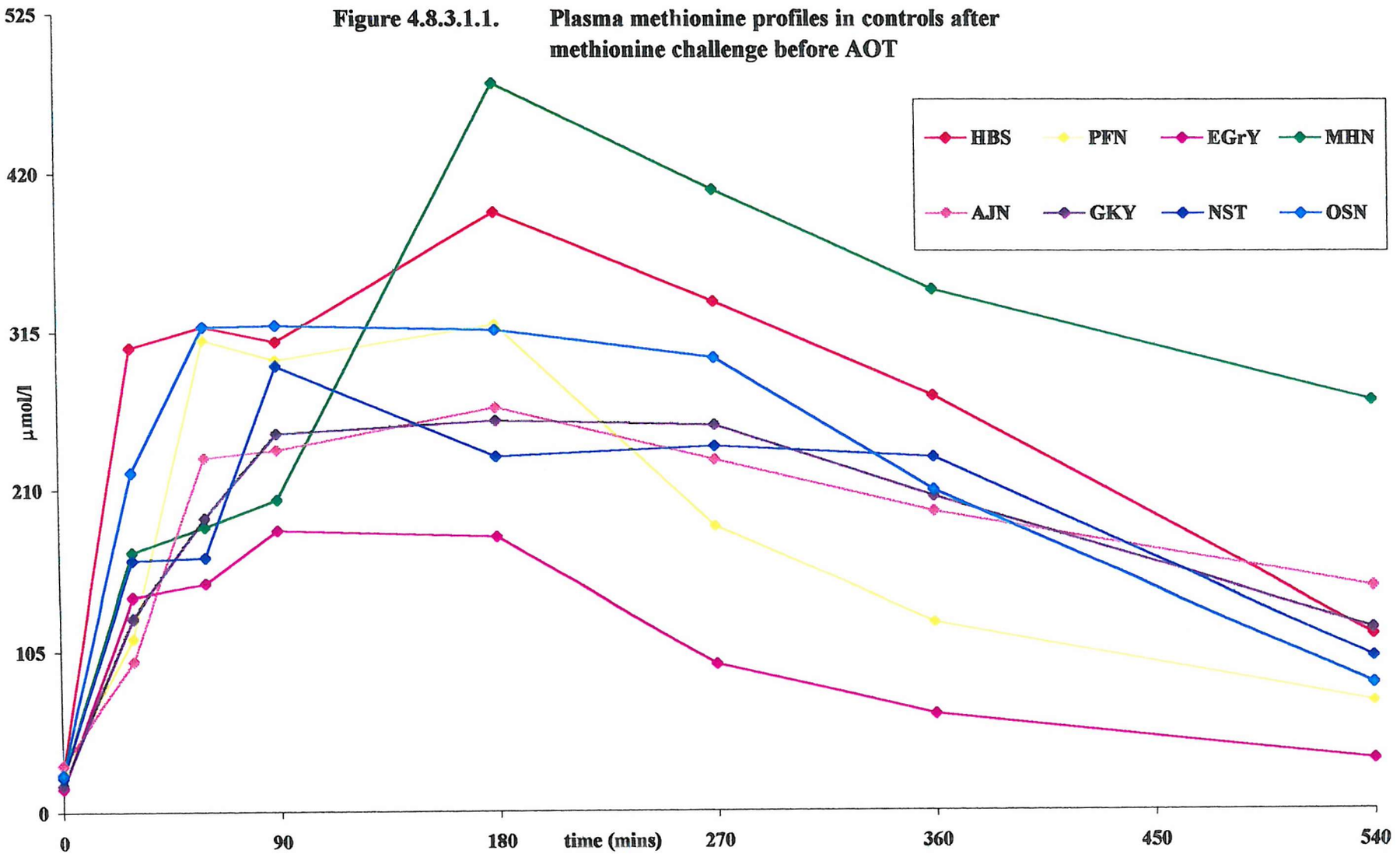


Figure 4.8.3.1.2. **Plasma methionine profiles in RAP after methionine challenge before AOT**

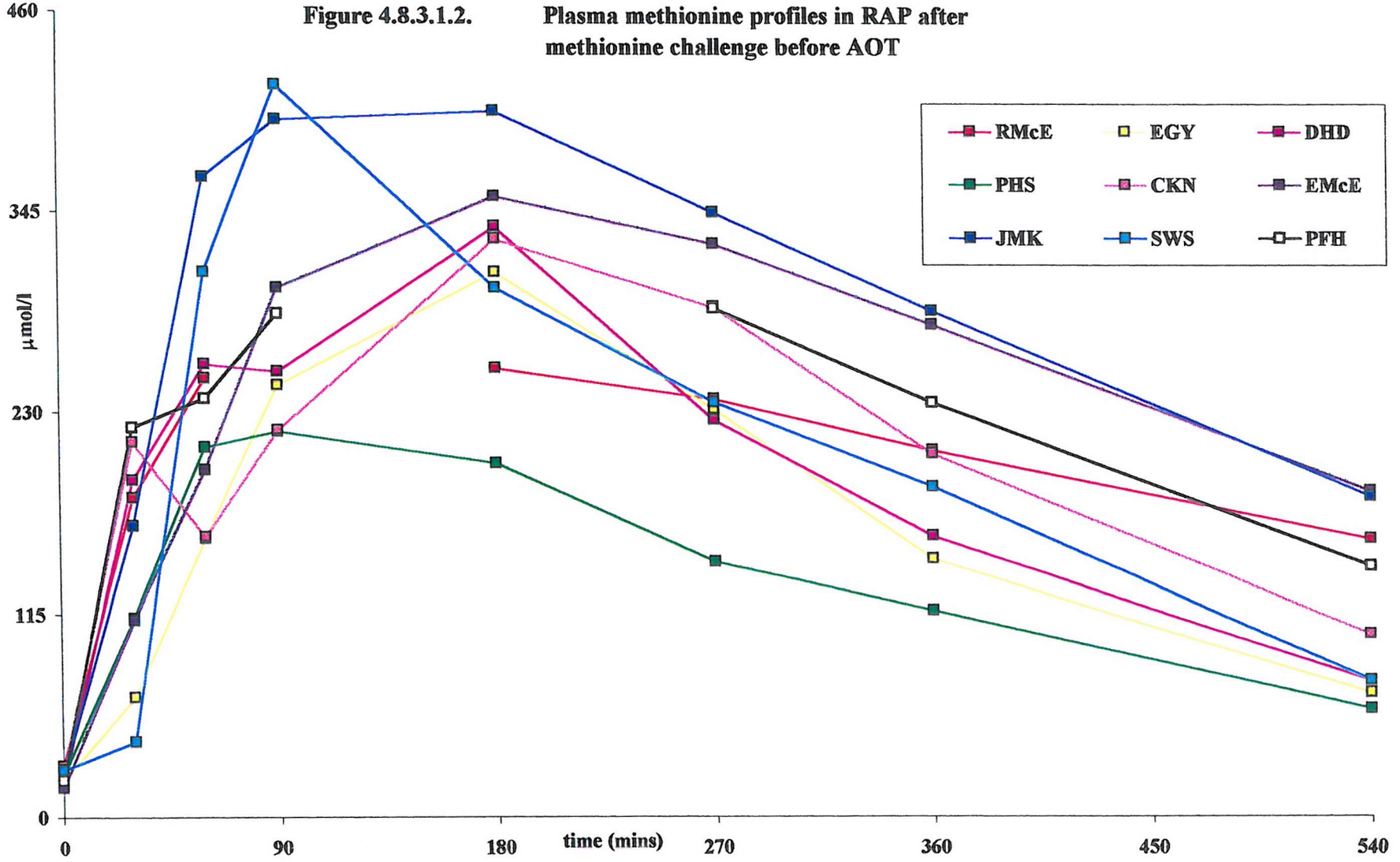


Figure 4.8.3.1.3. **Plasma methionine profiles in CP after methionine challenge before AOT**

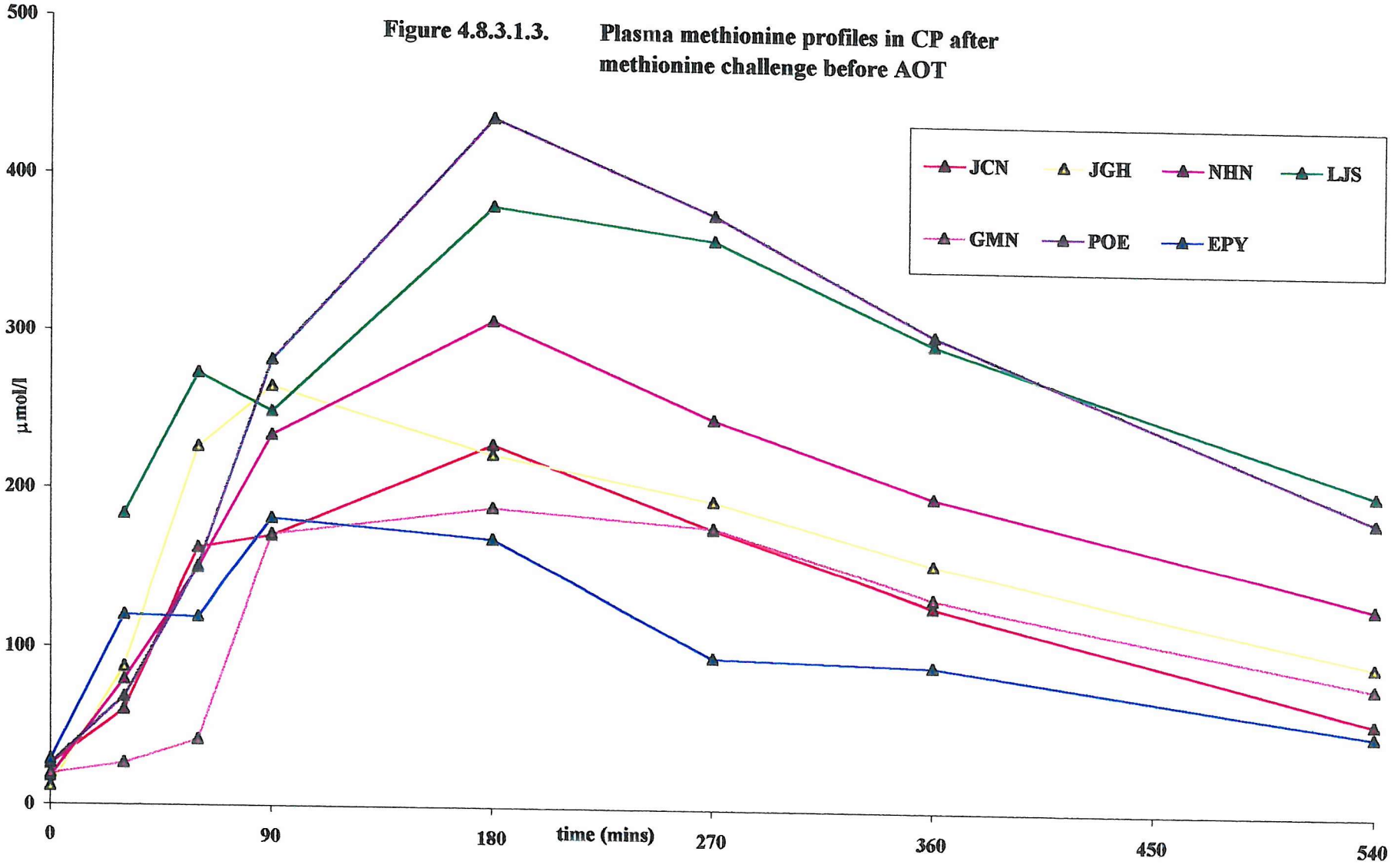


Figure 4.8.3.1.4. **Plasma methionine profiles in RAP after methionine challenge after AOT**

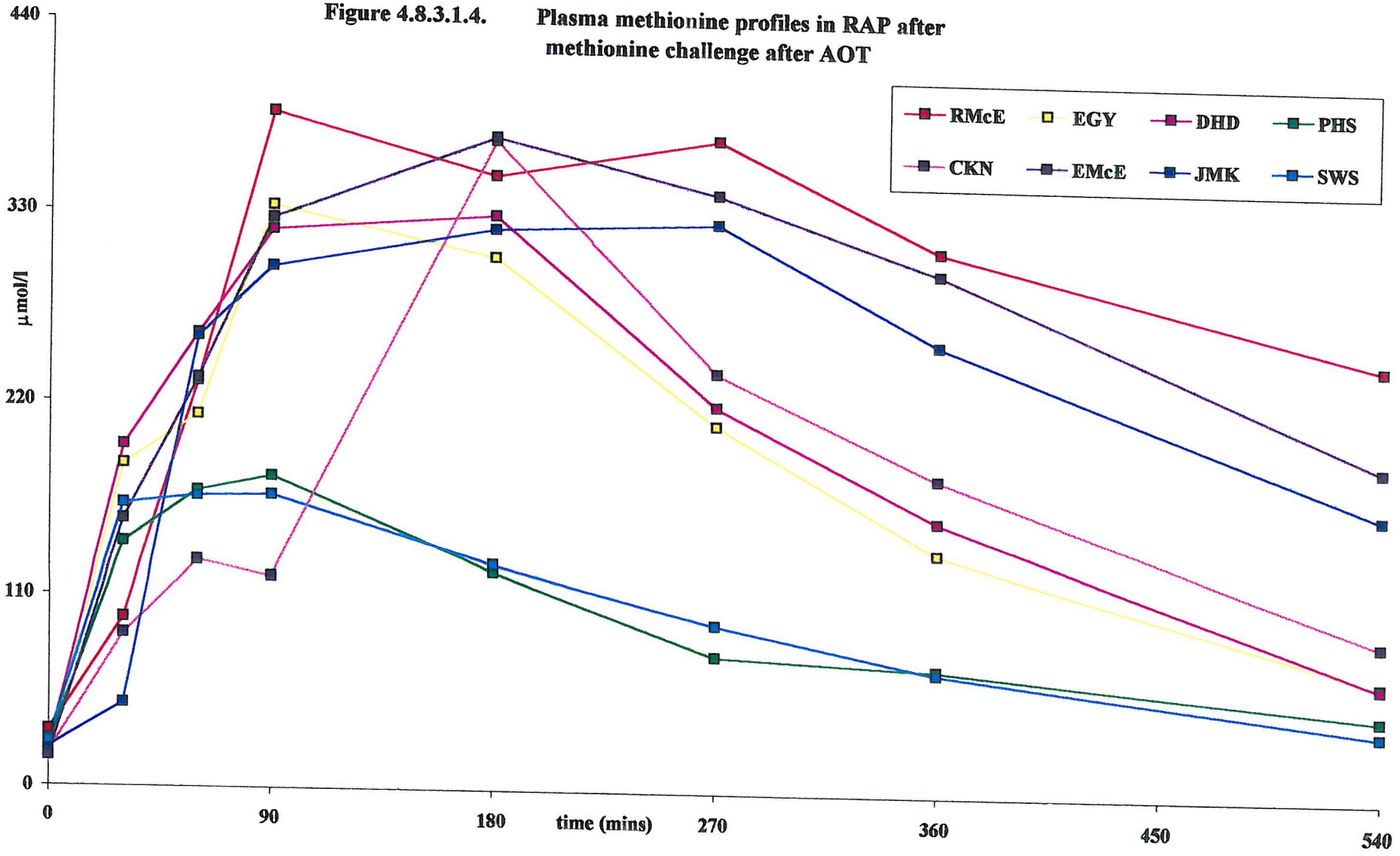


Figure 4.8.3.1.5. **Plasma methionine profiles in CP after methionine challenge after AOT**

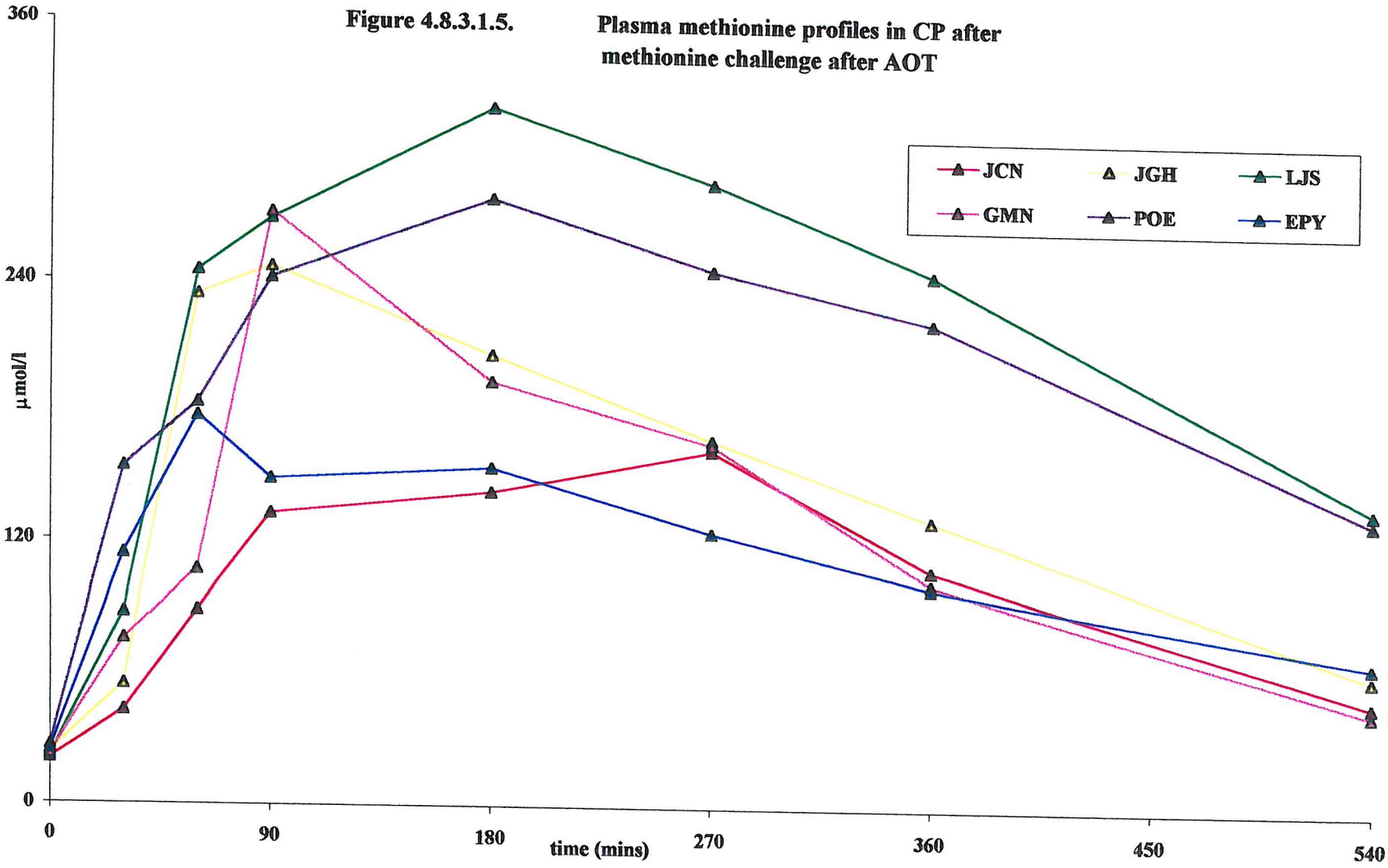


Figure 4.8.3.1.6. **Group mean (+/- 1s.d.) plasma methionine profiles after methionine challenge before AOT**

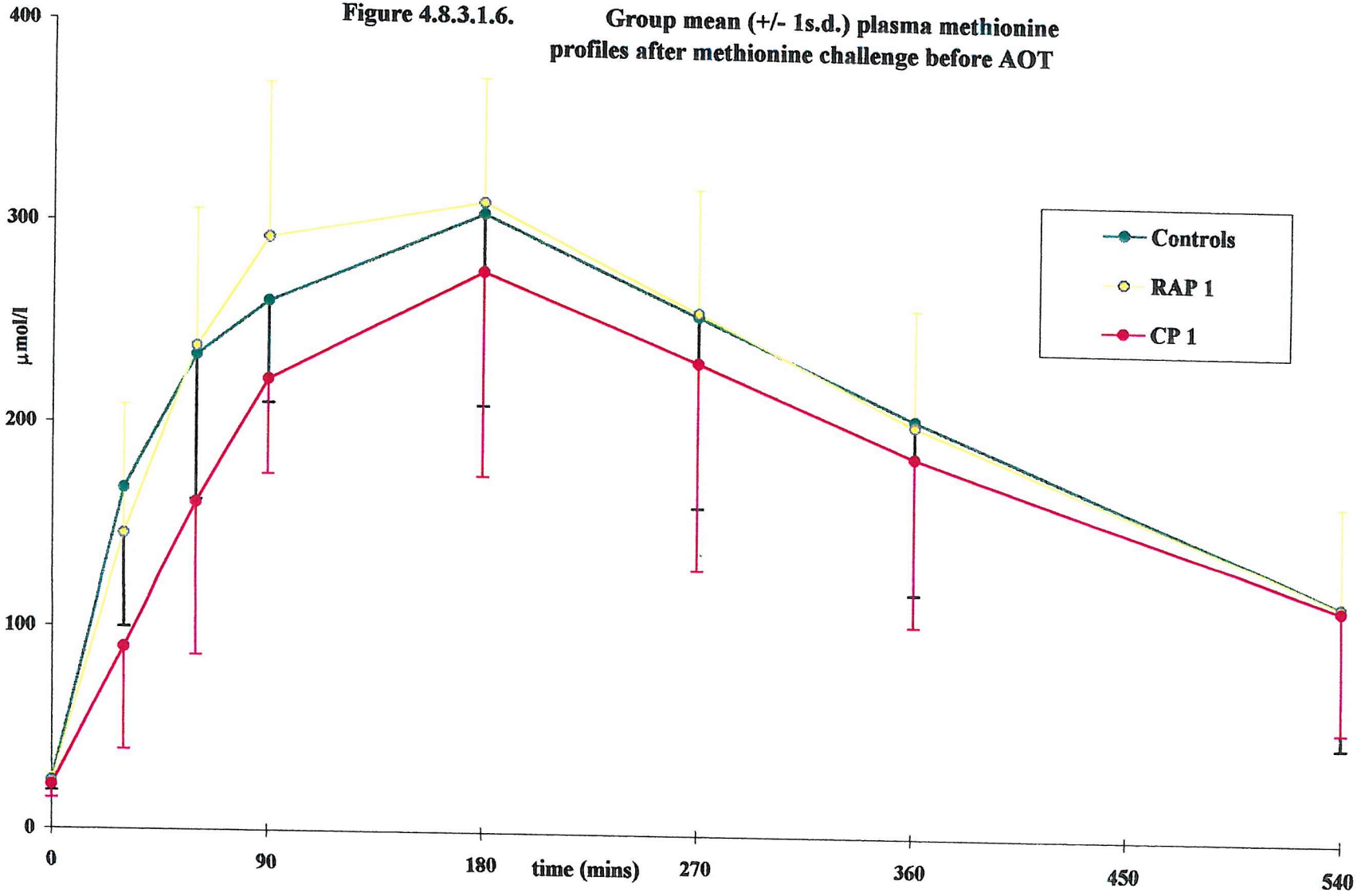
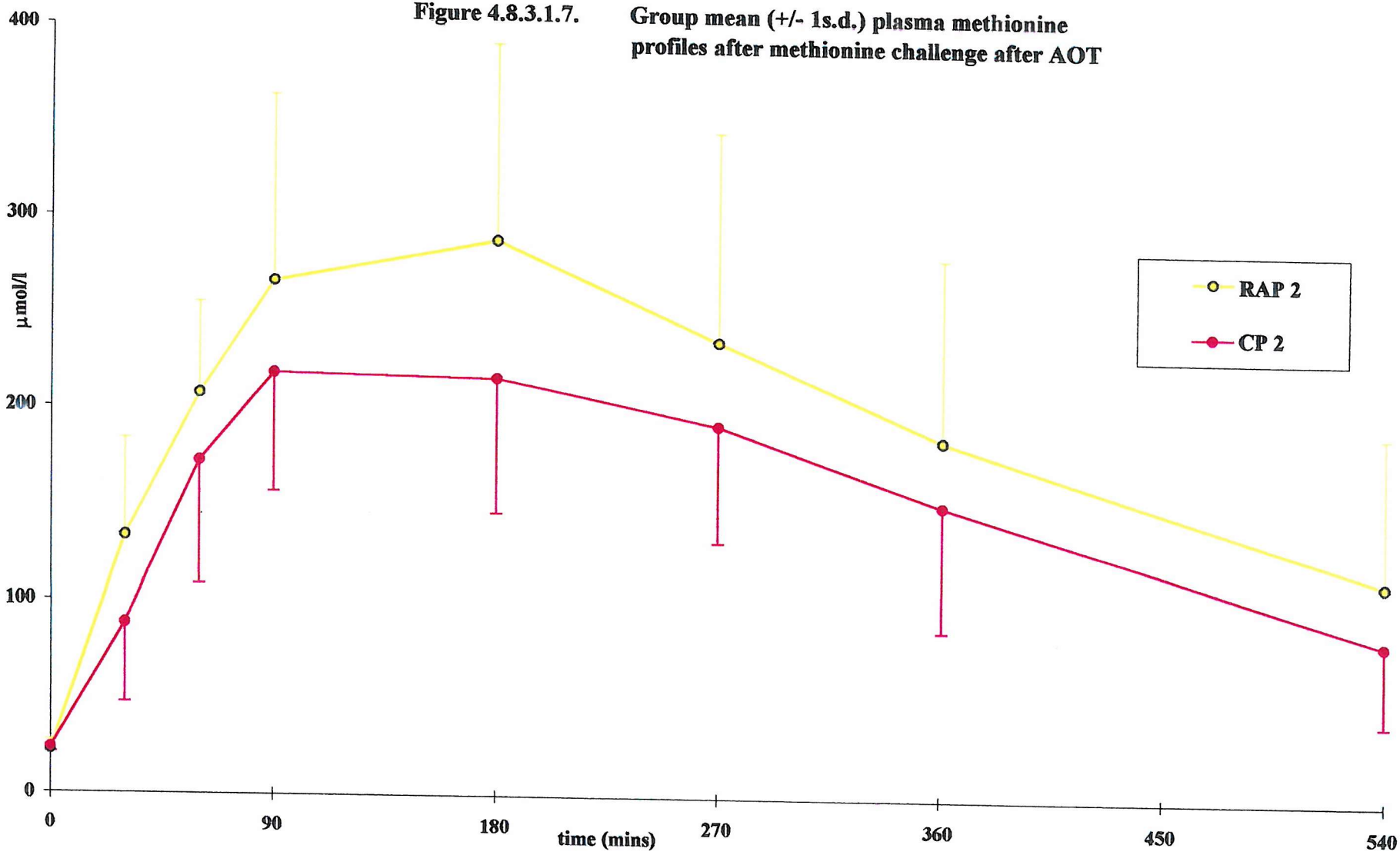


Figure 4.8.3.1.7. **Group mean (+/- 1s.d.) plasma methionine profiles after methionine challenge after AOT**



	Before AOT	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	AUC
	Time (mins)	0	30	60	90	180	270	360	540	μmol/l/hr
Control	mean	23.63	167.50	234.13	261.50	306.13	256.75	206.88	118.63	1955.9
	sd	4.84	67.45	71.86	50.75	95.43	94.78	85.54	70.61	584.7
RAP	mean	23.11	145.22	238.44	293.00	311.75	258.89	203.89	118.33	2002.4
	sd	3.92	63.89	68.10	76.81	61.59	61.02	58.15	49.83	425.4
CP	mean	21.86	90.00[a]	161.00	222.71	277.57	234.00	188.14	116.57	1719.2
	sd	6.34	50.25	74.38	47.09	101.66	103.03	82.85	60.80	647.3
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
	Time (mins)	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	
RAP	mean	22.34	133.00	207.56	266.78	289.13	237.00	186.81	114.84	1824.4
	sd	5.04	50.95	47.74	96.97	102.89	109.59	95.23	77.40	691.1
CP	mean	23.67	88.33[b]	172.83	218.67	216.83[c]	193.33	153.17	84.17	1441.3
	sd	2.16	40.72	63.97	61.51	69.88	60.55	64.72	42.10	466.6

[a] p = 0.03 with respect to control group at same time point (2-tailed Students t-test)

[b] p = 0.02 with respect to control group at same time point (2-tailed Students t-test)

[c] p = 0.02 with respect to RAP group at same time point before AOT (2-tailed Students t-test)

Table 4.8.3.1.3. **Group mean plasma methionine concentration (μmol/l) after oral methionine load before and after AOT (genders combined)**

Figure 4.8.3.1.8. **Plasma methionine profile in controls (group means +/- 1sd)**

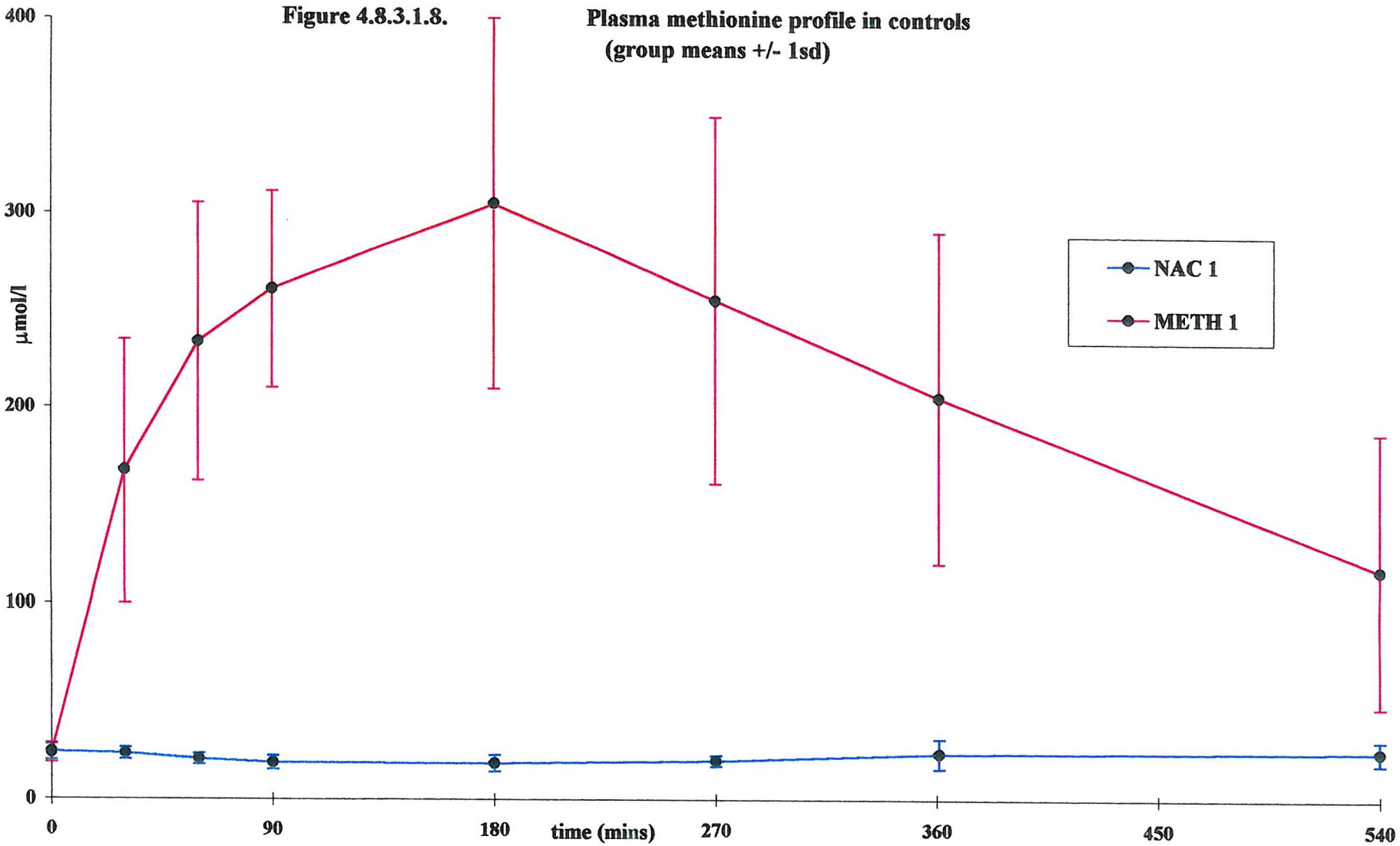


Figure 4.8.3.1.9. **Plasma methionine profiles in RAP (group means +/- 1sd)**

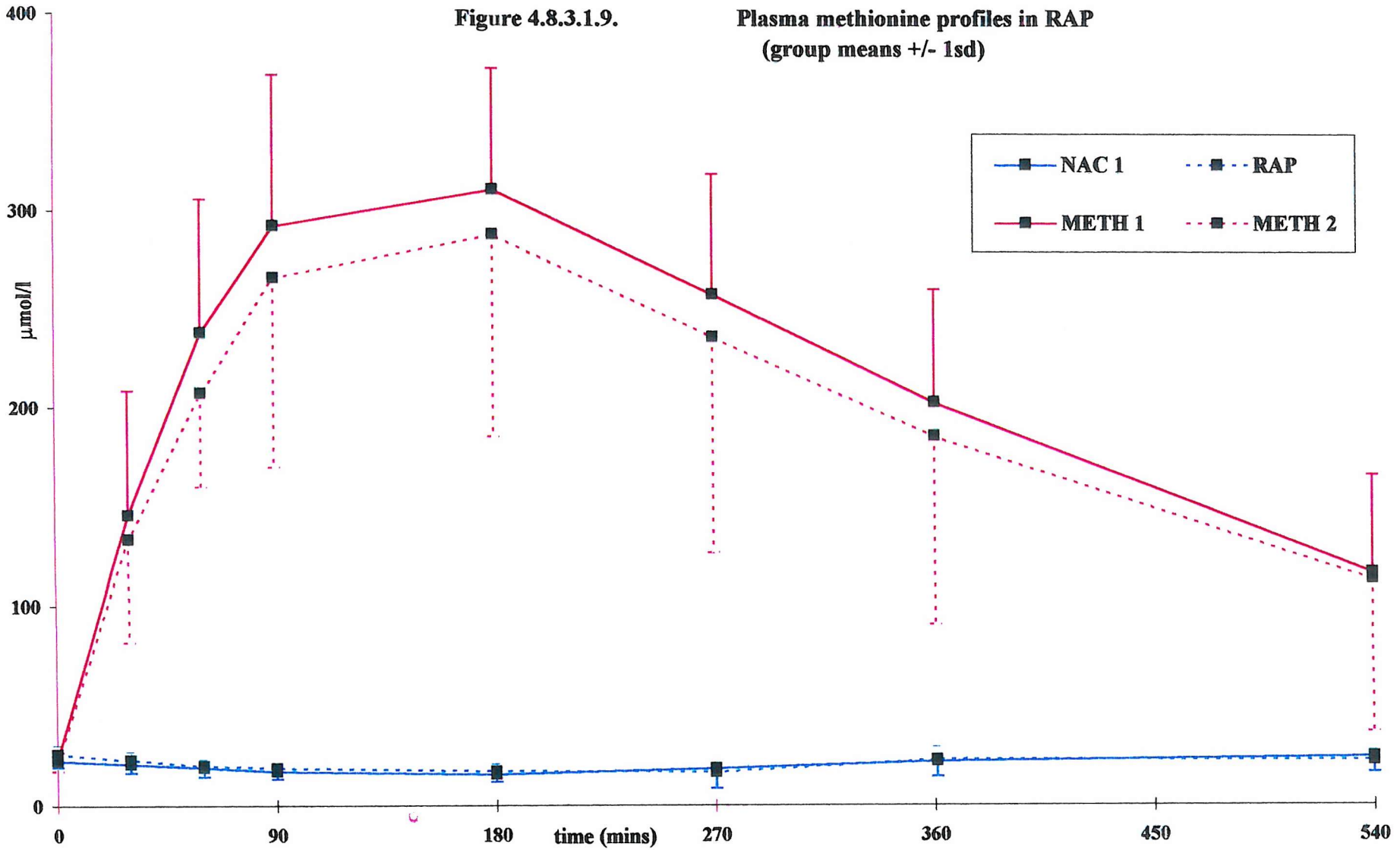


Figure 4.8.3.1.10. **Plasma methionine profile in CP (group means +/- 1sd)**

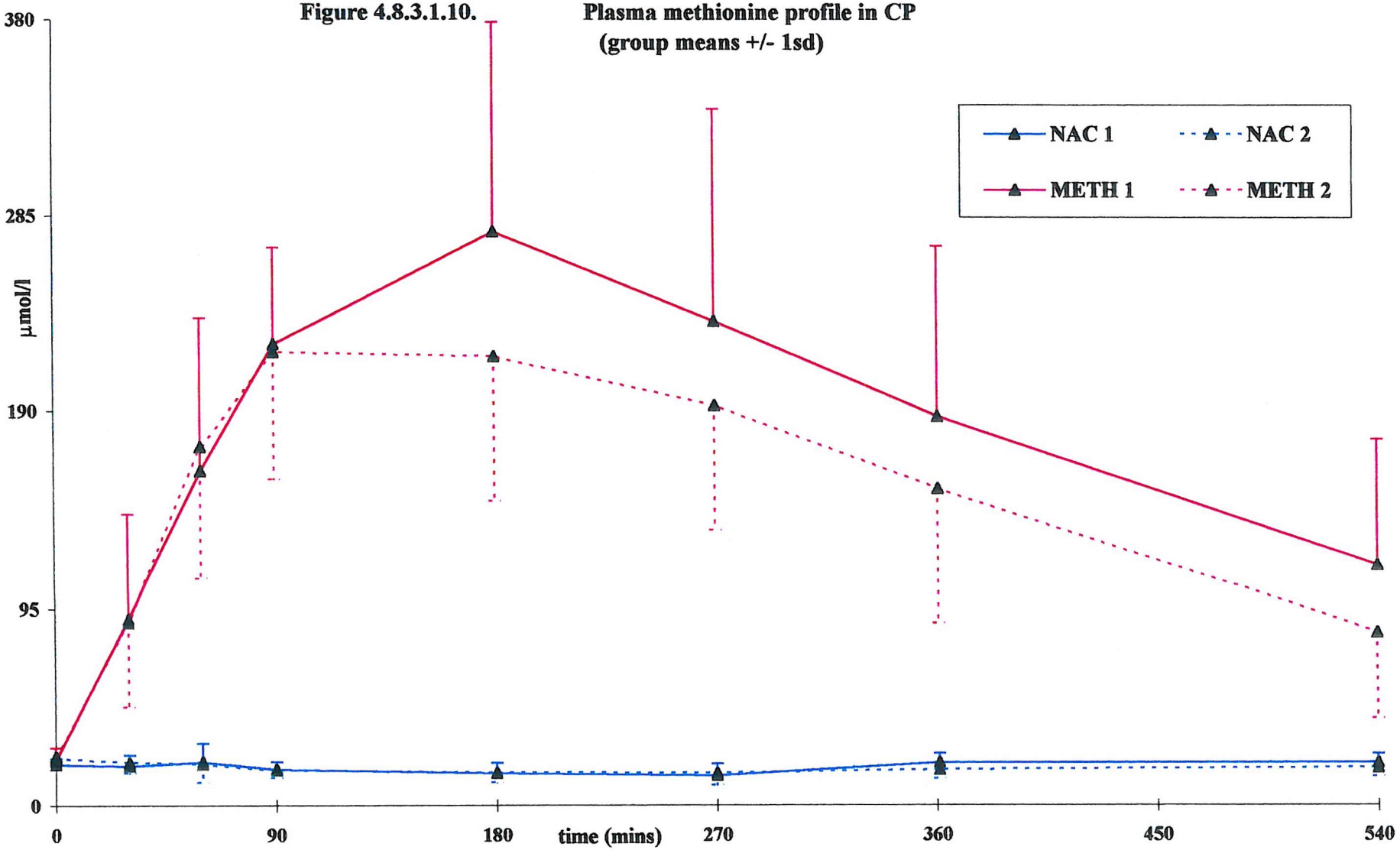


Figure 4.8.3.1.11. **Mean AUC plasma methionine**

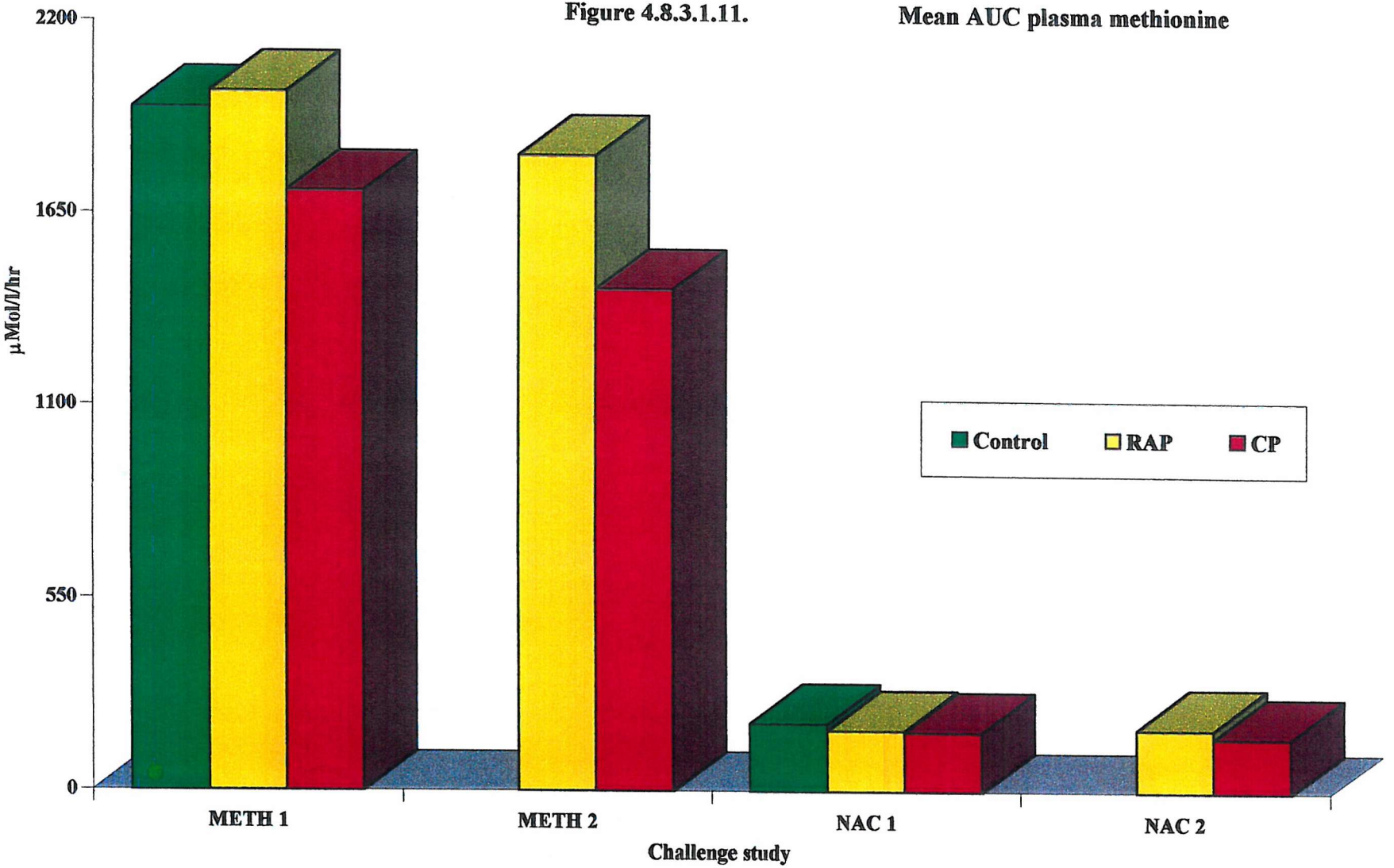


Figure 4.8.3.1.12. **Methionine group data means +/- 1sd**

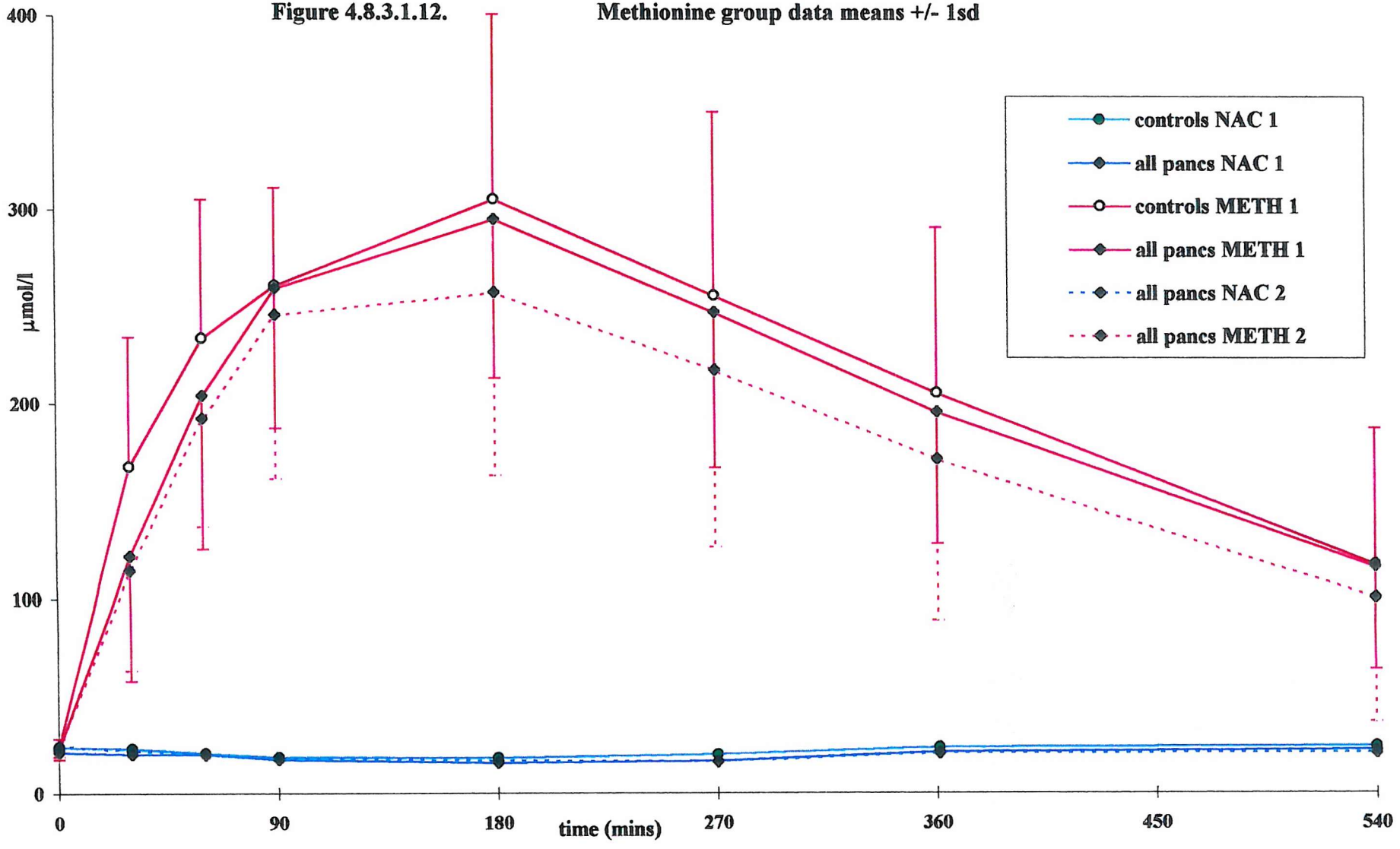
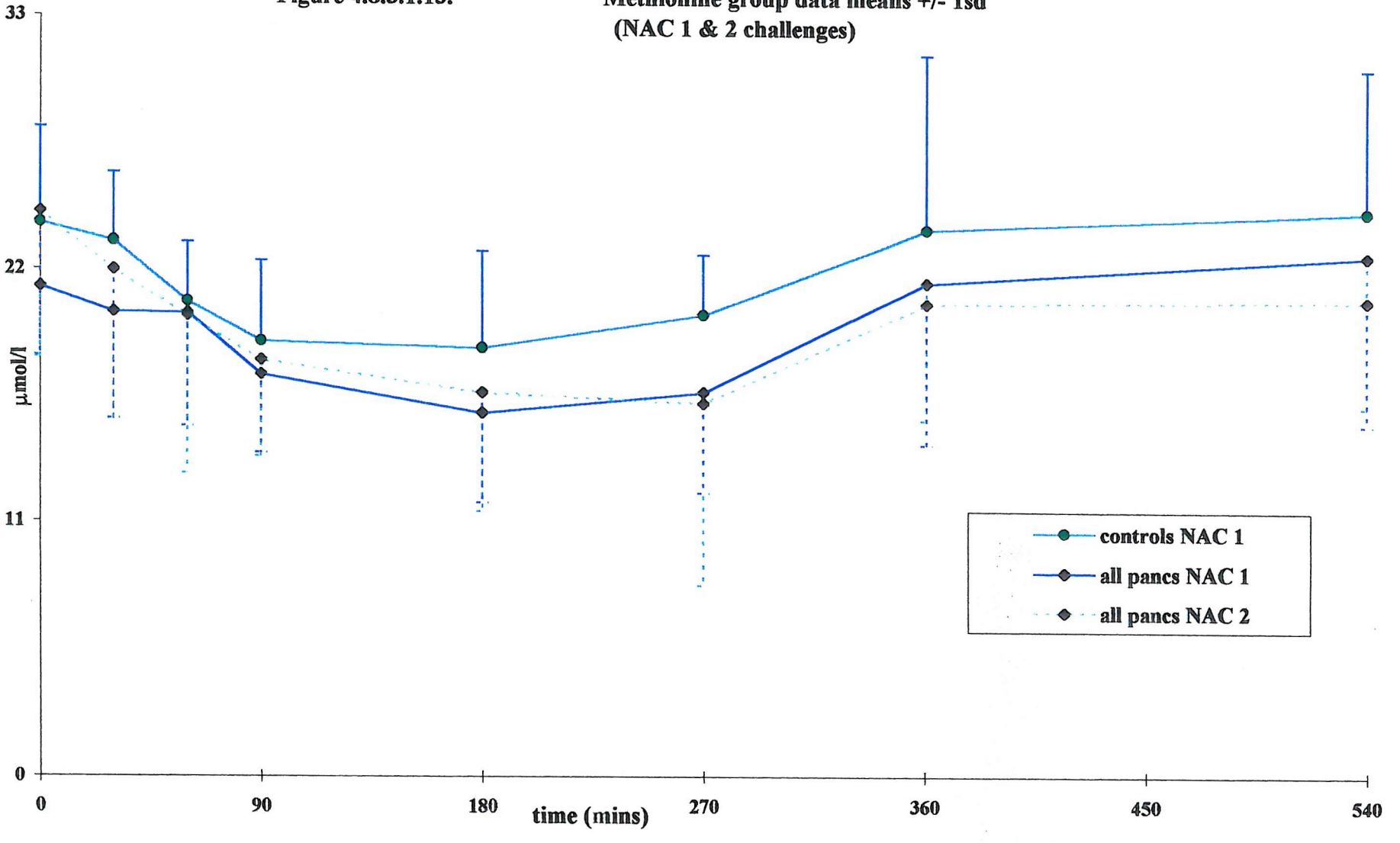


Figure 4.8.3.1.13. **Methionine group data means +/- 1sd (NAC 1 & 2 challenges)**

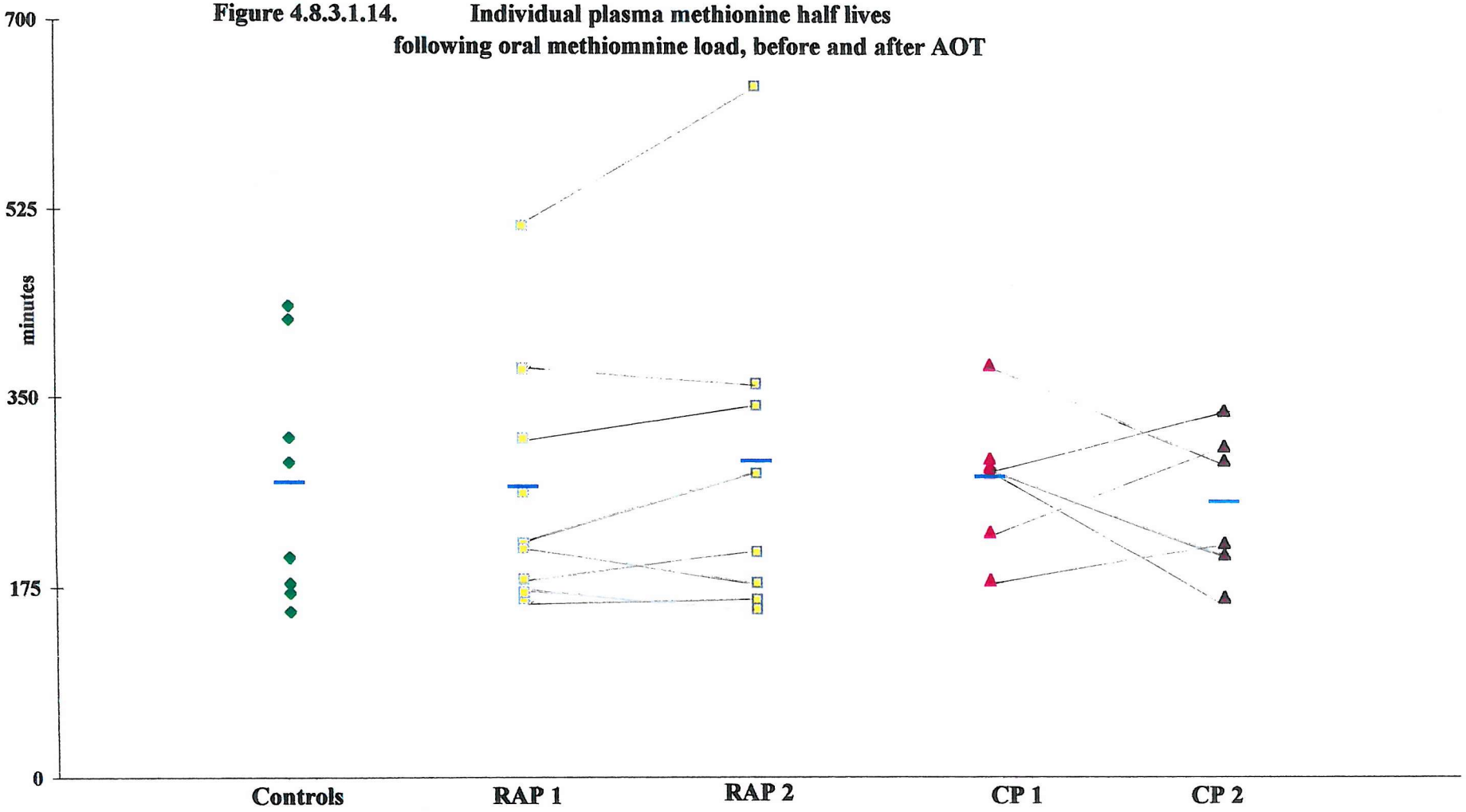


group/CS time period	c METH1 180-540	rap METH1 180-540	cp METH1 180-540	rap METH2 180-540	cp METH2 180-540
Individual No.					
1	201.59	511.27	183.67	640.92	218.31
2	170.91	165.86	288.03	165.28	207.15
3	153.99	171.70	296.57	156.70	
4	436.22	215.36	383.59	280.82	295.12
5	423.67	210.04	282.89	180.39	168.35
6	313.68	377.43	289.63	364.56	341.82
7	290.22	313.20	227.89	344.60	308.35
8	179.20	183.2		207.78	
9		262.34			
mean	271.18	267.82	278.90	292.63	256.52
sd	113.23	115.25	62.12	162.04	68.01
n	8	9	7	8	6
square root n	2.83	3.00	2.65	2.83	2.45
sem	40.03	38.42	23.48	57.29	27.76
mean	allpanc 1	272.67	allpanc 2	277.15	
sd		93.06		127.52	

	Statistics	**t-test**
METH 1	c v rap	0.95
	c v cp	0.87
	c v all p	0.97
	rap v cp	0.81
METH 2	c METH1 v rap METH2	0.76
	c METH1 v cp METH2	0.77
	rap METH2 v cp METH2	0.58
	c METH1 v all p METH2	0.91
	rap METH1 v rap METH2	0.72
	cp METH1 v cp METH2	0.60
	all p METH1 v all p METH2	0.91

Table 4.8.3.1.4. **Individual and group plasma methionine half lives (minutes) following an oral load**

Figure 4.8.3.1.14. Individual plasma methionine half lives following oral methiomnine load, before and after AOT



Methionine Volume of Distribution (Vd) and Metabolic Clearance Rate (MCR) after Methionine Loading **Table 4.8.3.1.5.**

		Meth1 Vd	Meth 1 MCR	Meth 2 Vd	Meth 2 MCR
	initials	**180-540**	**180-540**	**180-540**	**180-540**
Control	HBS	35.36	172.77		
	PFN	41.16	124.14		
	EGrY	53.26	175.63		
	MHN	50.60	75.29		
	AJN	71.27	115.64		
	GKY	60.87	173.02		
	NST	60.36	261.86		
	OSN	29.61	171.12		
	mean	*50.31*	*158.68*		
	sd	*14.12*	*55.41*		
RAP	RMcE	99.98	143.49	74.38	80.43
	EGY	34.56	144.97	36.24	151.96
	DHD	39.09	153.83	36.16	159.94
	PHS	74.87	257.08	148.16	365.63
	CKN	31.91	125.72	27.99	107.51
	EMcE	57.28	120.78	57.08	108.51
	JMK	33.62	81.51	42.06	84.59
	SWS	40.55	204.46	110.51	368.59
	PFH	38.58	113.52		
	mean	*50.05*	*149.49*	*66.57*	*178.39*
	sd	*23.30*	*52.41*	*42.55*	*119.87*
CP	JCN	49.01	217.35	78.09	247.89
	JGH	60.29	161.59	55.47	185.57
	NHN	48.73	111.63		
	LJS	48.70	97.44	51.13	120.06
	GMN	58.82	167.10	44.61	183.62
	POE	35.48	92.57	55.38	112.28
	EPY	94.01	264.60	105.80	237.79
	mean	*56.43*	*158.90*	*65.08*	*181.20*
	sd	*18.47*	*64.65*	*22.93*	*56.86*

	ttest	ttest
	Vd	**MCR**
p values	**180-540**	**180-540**
c v rap 1	0.98	0.73
c v cp 1	0.49	0.99
rap1 V rap2	0.28	0.38
cp1 v cp2	0.31	0.15
c v allpanc 1	0.73	0.84
c v allpanc 2	0.15	0.52
allpanc 1 v allpanc 2	0.21	0.36

Where: Volume distribution - litres MCR - ml / minute

Figure 4.8.3.1.15. Plasma methionine profiles of controls in NAC 1 challenge

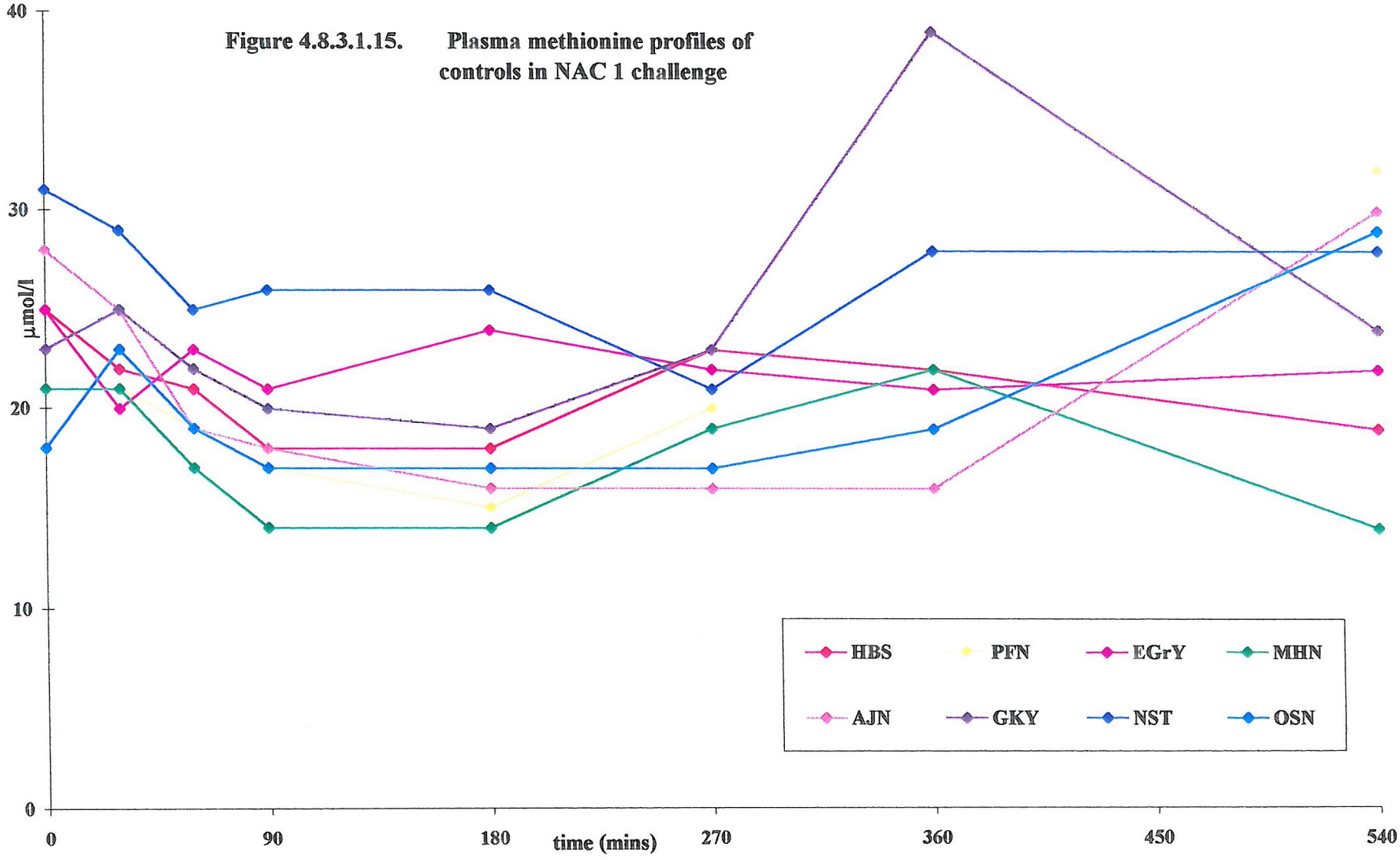


Figure 4.8.3.1.16. **Plasma methionine profiles in RAP in NAC 1 challenge**

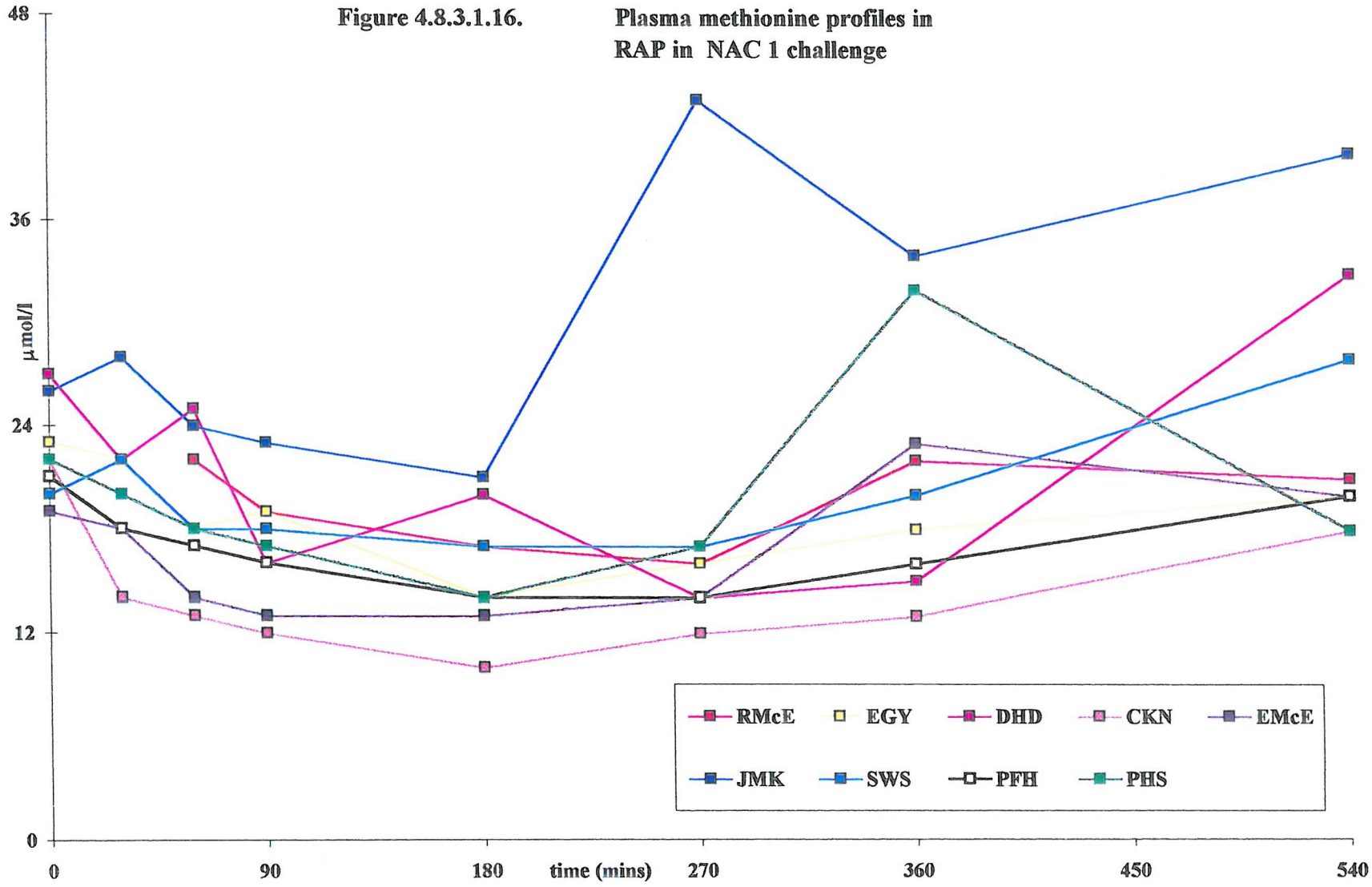


Figure 4.8.3.1.17. **Plasma methionine profiles in CP in NAC 1 challenge**

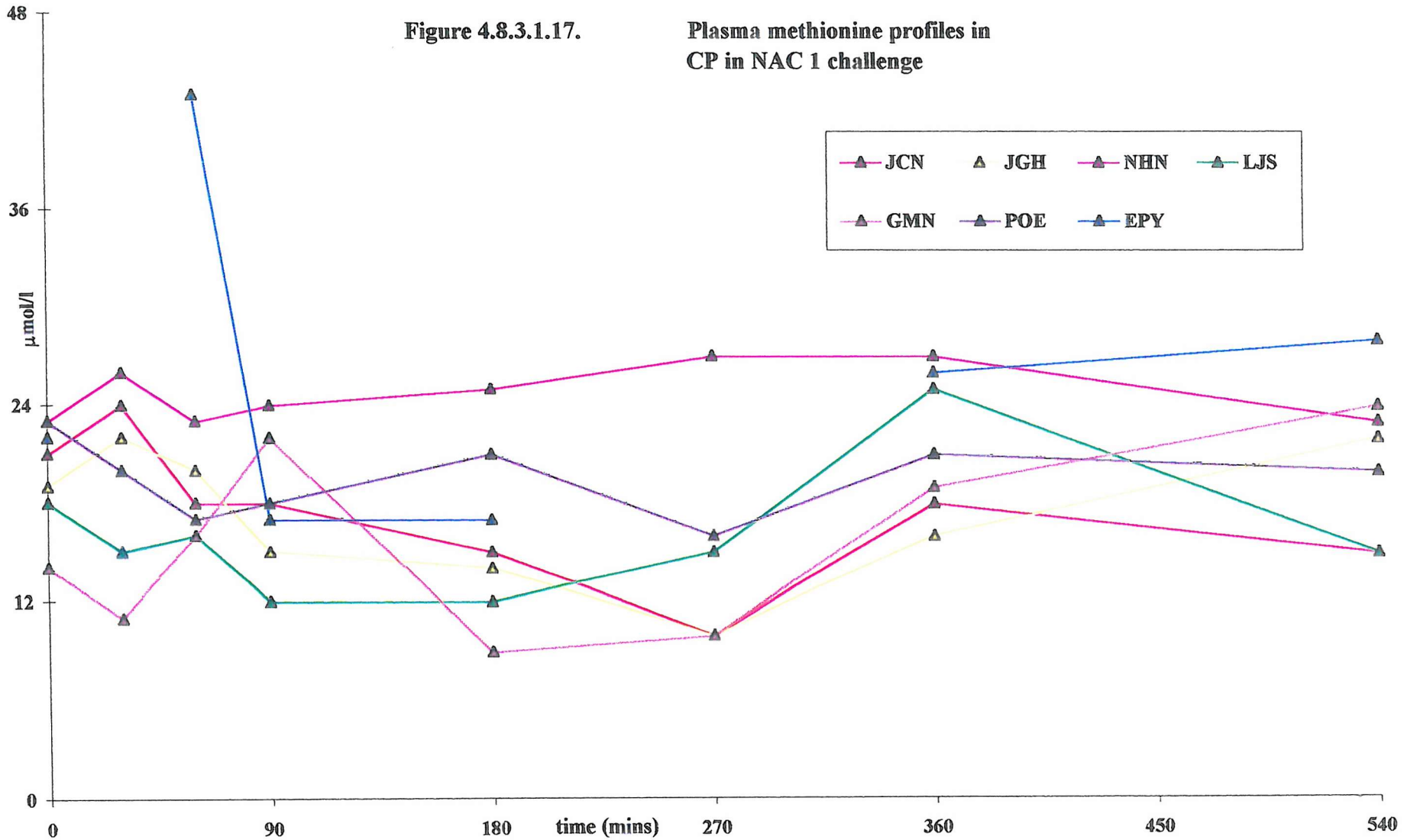


Figure 4.8.3.1.18. Plasma methionine profiles in RAP in NAC2 challenge

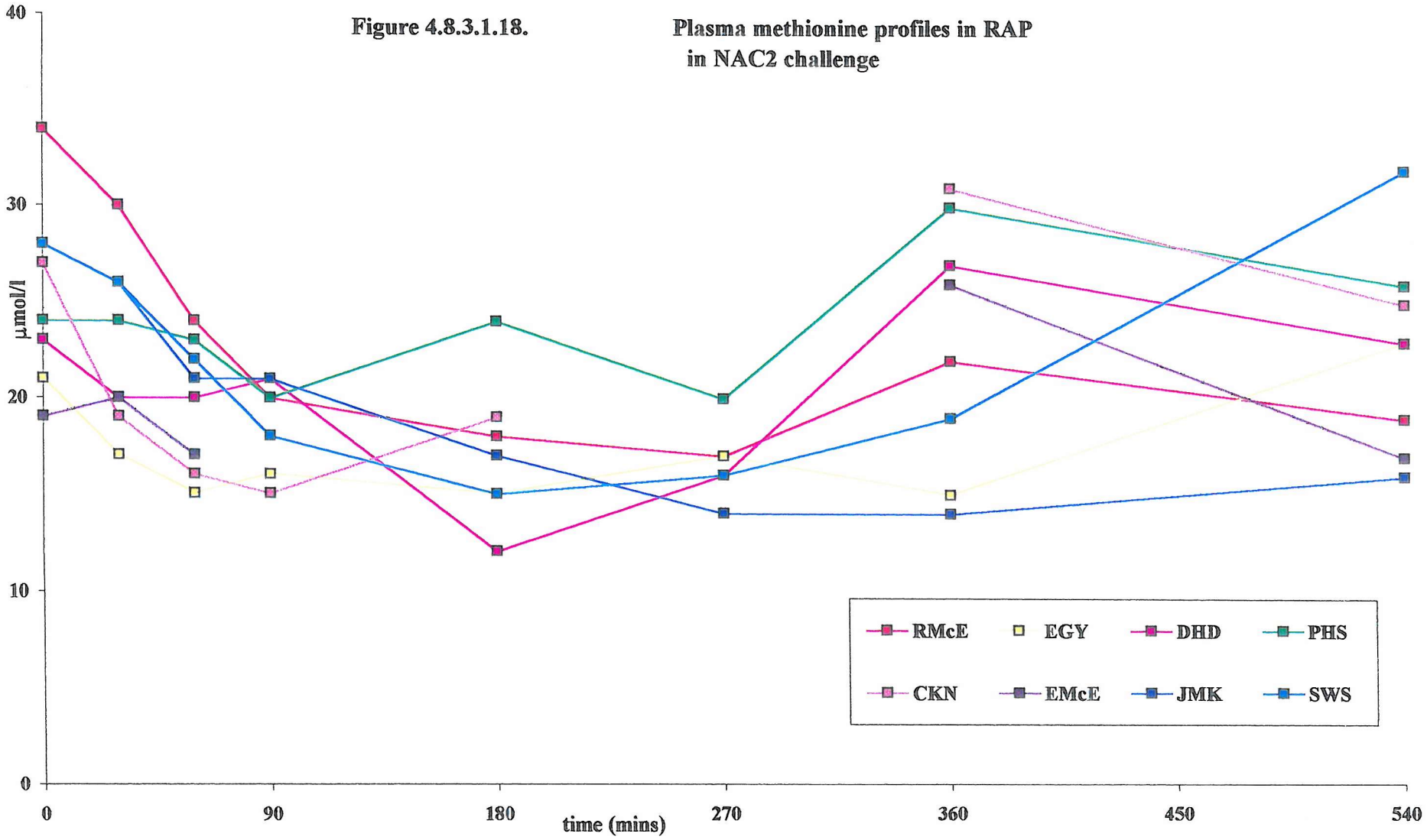


Figure 4.8.3.1.19. **Plasma methionine profiles in CP in NAC2 challenge**

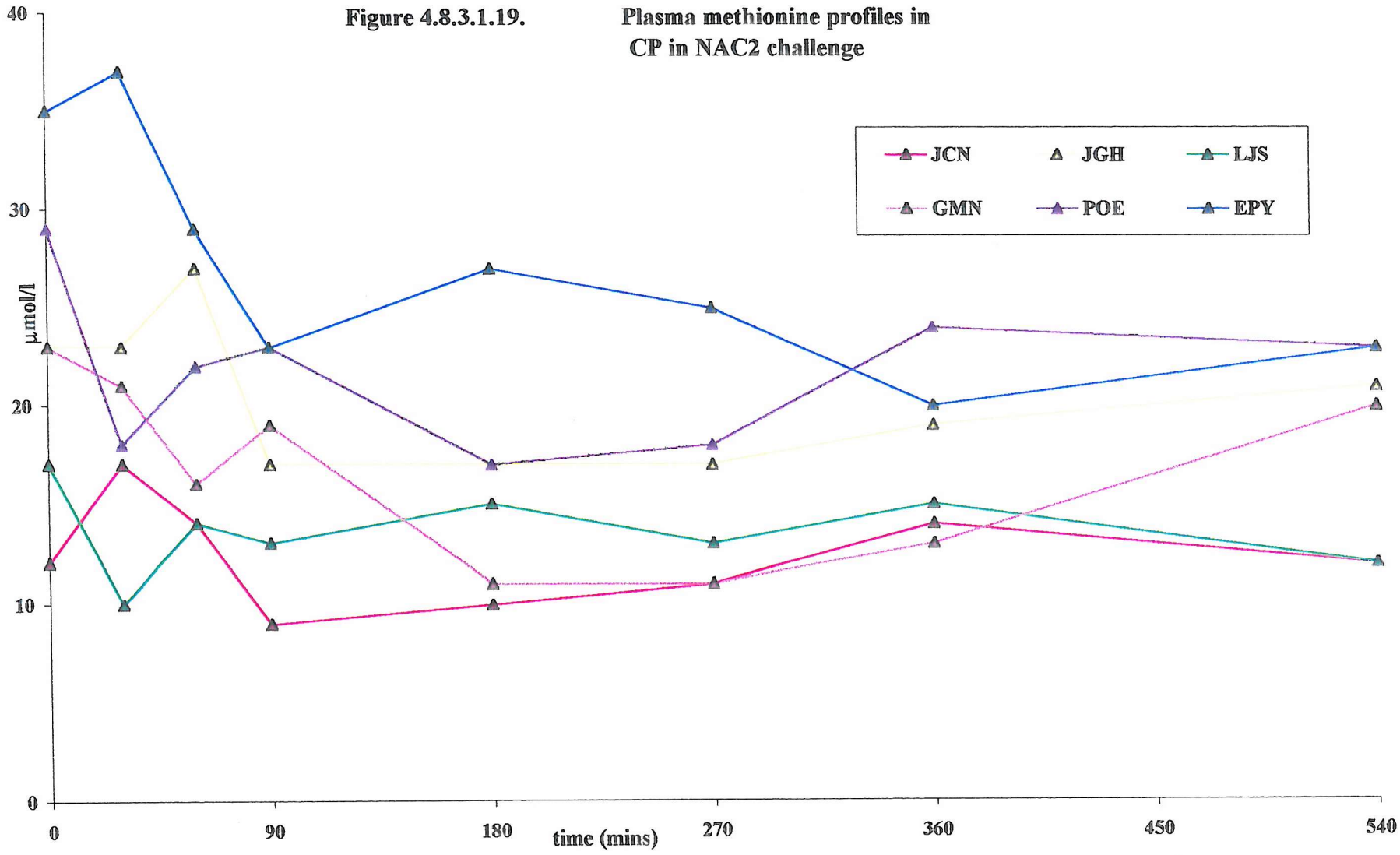


Figure 4.8.3.1.20. **Group mean (+/- 1s.d.) plasma methionine profiles after NAC1 challenge**

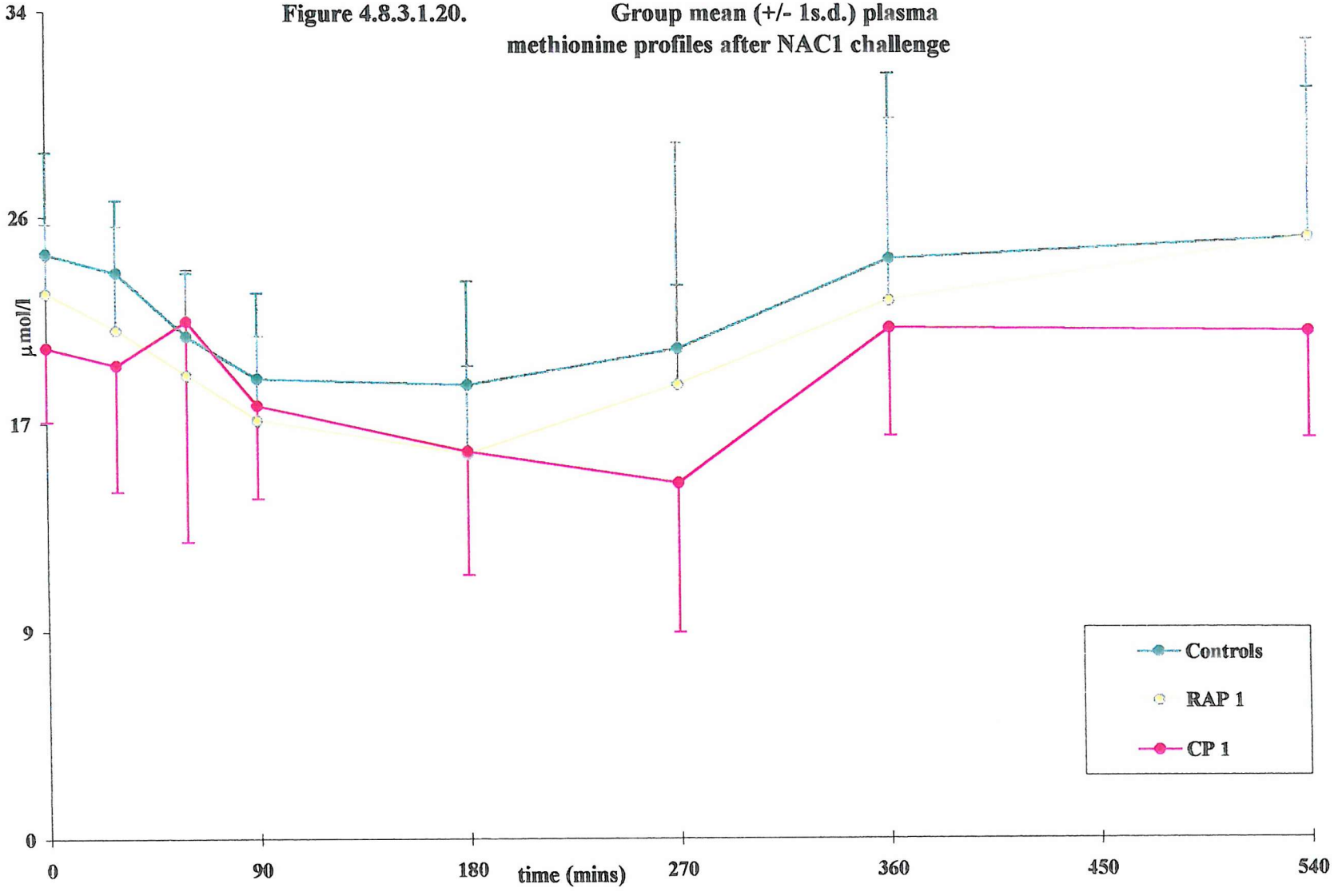
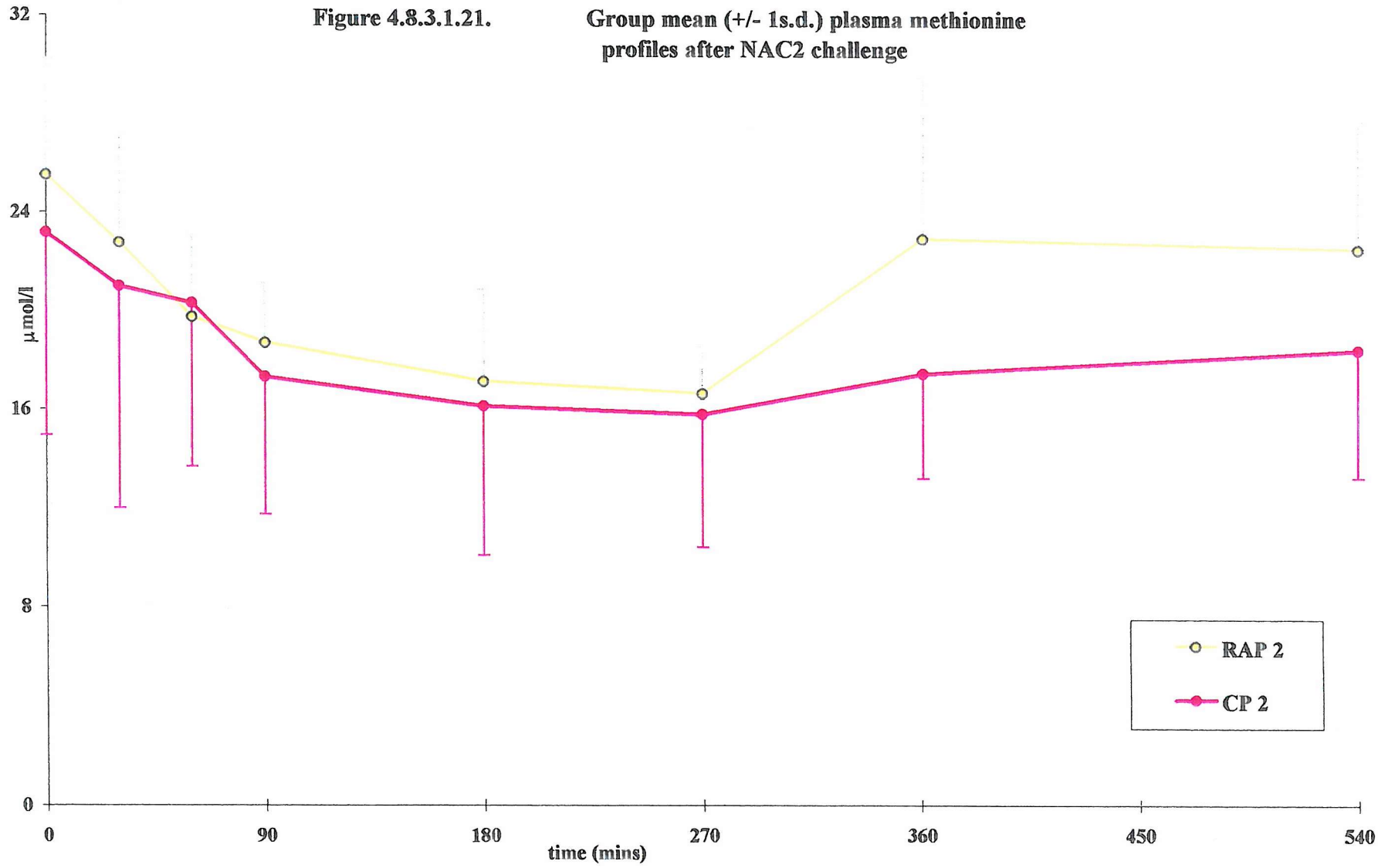


Figure 4.8.3.1.21. **Group mean (+/- 1s.d.) plasma methionine profiles after NAC2 challenge**



	Before AOT	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	AUC
	Time (mins)	0	30	60	90	180	270	360	540	μmol/l/hr
Control	mean	24.00	23.25	20.63	18.88	18.63	20.13	23.86	24.75	195.80
	sd	4.17	2.96	2.62	3.56	4.27	2.64	7.60	6.16	27.80
RAP	mean	22.22	20.50	18.78	17.00	15.56	18.11	21.44	24.22	177.30
	sd	2.73	4.11	4.15	3.32	3.50	9.48	7.32	7.76	46.50
CP	mean	20.00	19.67	21.86	18.00	16.14	14.67	21.71	21.00	172.70
	sd	3.27	5.68	9.65	4.04	5.43	6.62	4.31	4.76	36.00
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
	Time (mins)	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	**AUC**
RAP	mean	25.50	22.75	19.75	18.71	17.14	16.29[a]	23.00	22.63	184.00
	sd	4.75	4.43	3.37	2.43	3.80	2.06	6.55	5.26	22.40
CP	mean	23.17	21.00	20.33	17.33	16.17	15.83	17.50	18.50	158.90
	sd	8.21	9.01	6.65	5.57	6.08	5.38	4.23	5.17	43.90

[a] $p = 0.008$ with respect to control group at same time point (2-tailed Students t-test)

Table 4.8.3.1.6. Group mean plasma methionine concentrations (μmol/l) after oral NAC load before and after AOT

	Time points compared						
t-test comparisons	**0 v 30**	**0 v 60**	**0 v 90**	**0 v 180**	**0 v 270**	**0 v 360**	**0 v 540**
Control pre-AOT	0.528	0.018	**0.001**	**0.004**	0.046	0.865	0.771
RAP pre-AOT	0.968	**0.009**	**0.002**	**1.E-04**	0.185	0.765	0.359
CP pre-AOT	0.341	0.055	0.008	**0.002**	0.311	0.745	0.440
RAP post-AOT	0.026	**0.003**	**0.004**	**0.003**	**0.007**	0.441	0.278
CP post-AOT	0.411	0.207	**0.007**	0.013	0.009	0.069	0.041
All subjects	0.014	**0.002**	**3.E-09**	**2.E-11**	**4.E-06**	0.238	0.613

Table 4.8.3.1.7. **Students t-test comparison of plasma methionine following oral NAC loading**

4.8.3.2. Plasma cystine

Mean fasting plasma cystine concentrations in the three subject groups (controls, recurrent acute and chronic pancreatitis) did not differ, prior to or after AOT, Figure 4.8.3.2.1. When the individuals were separated by gender, no difference in fasting plasma cystine concentrations was shown, Table 4.8.3.2.1, for although the comparison between cystine following NAC in women (RAP1 v CP1) gave p = 0.044, this is not significant after Bonferroni. Challenging subjects with NAC or methionine did not alter the subsequent baseline fasting plasma cystine concentration, a fortnight later, when the second challenge study was performed, Table 4.8.3.2.2.

		Phase 1	Phase 2	Phase 2
		Baseline	after methionine 1st	after NAC 1st
Control	*mean*	117.4	108.6	126.7
	sd	13.0	21.9	36.5
RAP	*mean*	123.0	129.0	129.5
	sd	22.9	19.5	23.3
CP	*mean*	110.0	117.0	139.7
	sd	21.5	43.8	11.6

Table 4.8.3.2.2. Baseline plasma cystine (μmol/l) mean and s.d. for groups in phase 1 and phase 2 divided by first challenge compound received.

Figures 4.8.3.2.2. - 4. collate the individual plasma cystine profiles of the subjects stratified by subject group, i.e. controls, RAP and CP, during the methionine challenge study before AOT, with Figure 4.8.3.2.5. showing the group mean values. Figures 4.8.3.2.6 - 7 plot the individual subjects results from the methionine challenge study after AOT, with Figure 4.8.3.2.8 showing the group mean values. Table 4.8.3.2.3. collates the group mean plasma cystine concentrations after oral methionine loading.

Figures 4.8.3.2.9. - 15. show the corresponding results for the NAC challenge studies before and after AOT. These graphs show that following an oral loading dose of NAC, an early rise in plasma cystine concentration occurs, data summarised

in Table 4.8.3.2.4. Following NAC loading, the group mean peak cystine concentration appears after 30 minutes in the control group and 90 minutes in patients with chronic pancreatitis. There was a difference in the peak concentration, at the 90 minute time-point between healthy controls and patients with CP, Table 4.8.3.2.4. The group of patients with a history of recurrent acute pancreatitis had mean peak cystine concentrations falling in between those of the controls and the CP patients but did not differ significantly from either group. AOT had no effect on peak levels or the profiles.

The mean AUC (μmol/l/hr)for plasma cystine concentrations in the study groups during the 4 challenge studies are shown in Figure 4.8.3.2.16. with actual values in Tables 4.8.3.2.3. - 4. No difference in AUC was confirmed at baseline or between the groups before or after AOT.

T½ values were calculated from the line of best fit, following first order kinetics, from the graphs of plasma cystine concentrations shown in Figures 4.8.3.2.9 - 11. and 4.8.3.2.13 - 14. Before AOT plasma cystine peaked in the control group 30, RAP at 60 and CP at 90 minutes after oral loading with NAC. After AOT RAP peaked at 90 and CP at 60 minutes. First order kinetics appeared to be exhibited between these peaks and either the 270 or 360 time point. The period between 90 and 360 minutes post dose was chosen to calculate t½. Mean plasma cystine t½ following an oral load of NAC (50mg/kg) are summarised in Table 4.8.3.2.5. with an average half life of between 318.1 - 480.8 minutes. No difference in cystine t½ was found between controls and patients with either RAP or CP.

In contrast to plasma methionine t½, the cystine calculations are confounded by high baseline levels, values at 540 minutes that are lower than baseline and relatively small increases after oral loading with NAC. These factors result in cystine kinetics not being strictly first order (levels decreasing towards zero). Furthermore, small difference in plasma cystine may be masked by imprecisions in analytical determinations of cystine. Vd and MCR have not been calculated because of these reasons for plasma cystine.

Summary of plasma cystine results in challenge study

- Fasting plasma cystine levels are normal in patients with pancreatic disease.
- Oral NAC results in elevation of plasma cystine, with a peak between 30 and 90 minutes.
- Oral methionine has no effect on plasma cystine levels.
- No difference was found after NAC loading in plasma cystine t½ or AUC, between healthy controls or the patient groups, either before or after AOT.

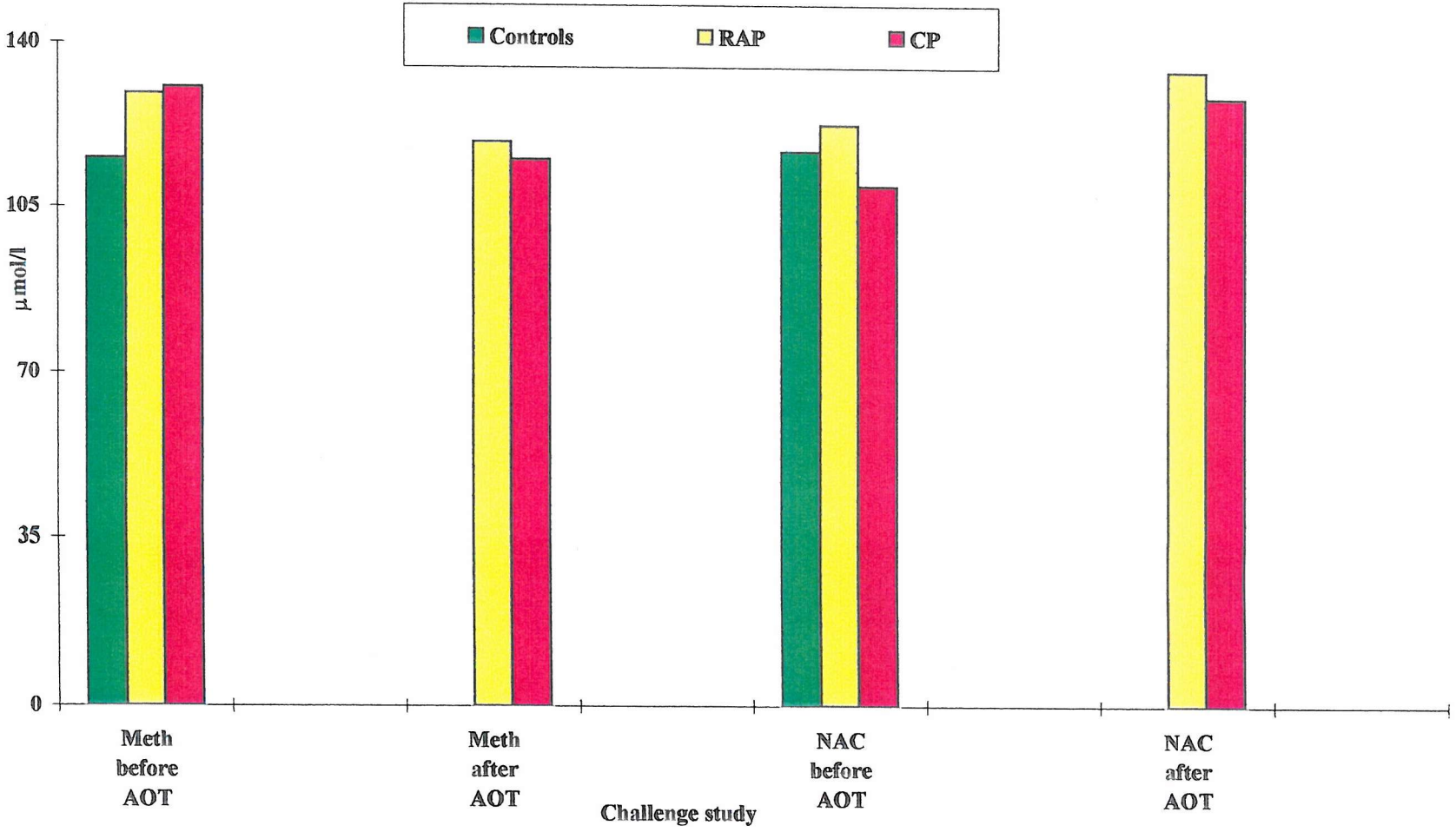


Figure 4.8.3.2.1. **Mean fasting baseline plasma cystine (µmol/l) in mixed gender groups in challenge studies.**

Phase		Control NAC	Control METH	RAP NAC	RAP METH	CP NAC	CP METH		t tests NAC	t tests METH
Before AOT	means	117.4	115.4	123.0	129.1	110.0	130.6	*c v cp*	0.449	0.345
Both genders	sd	13.0	27.2	22.9	18.5	21.5	26.5	*c v rap*	0.538	0.269
After AOT	means			134.9	119.3	129.2	115.6	*rap2 v cp2*	0.679	0.720
	sd			25.7	19.4	21.8	15.1	*rap1 v cp1*	0.264	0.919
Before AOT	means	120.4	119.6	118.4	133.0	120.5	126.3	*c v rap*	0.888	0.414
Male	sd	13.6	27.8	27.1	20.7	20.6	28.5	*c v cp*	0.994	0.928
After AOT	means			136.2	125.3	135.0	122.0	*rap1 v cp1*	0.462	0.965
	sd			23.2	19.6	29.7	5.7	*rap2 v cp2*	0.965	0.707
Before AOT	means	112.3	108.3	128.8	119.5	88.5	148.0	*c v rap*	0.220	0.602
Female	sd	12.7	30.6	18.3	9.2	12.0	N/A	*c v cp*	0.147	N/A
After AOT	means			132.7	111.3	127.0	90.0	*rap1 v cp1*	0.044	N/A
	sd			34.8	19.8	29.7	N/A	*rap2 v cp2*	0.860	N/A
	t tests	*c(m) v c(f)*		*rap(m) v rap(f)*		*cp(m) vs cp(f)*				
	Before AOT	0.439	0.629	0.518	0.292	0.083	N/A			
	After AOT			0.886	0.402	0.813	N/A			

Table 4.8.3.2.1. **Mean (1 s.d.) fasting baseline plasma cystine (μmol/l) in single- and mixed gender groups and t-test comparisons between groups.**

Figure 4.8.3.2.2. **Plasma cystine profiles of controls in METH 1 challenge**

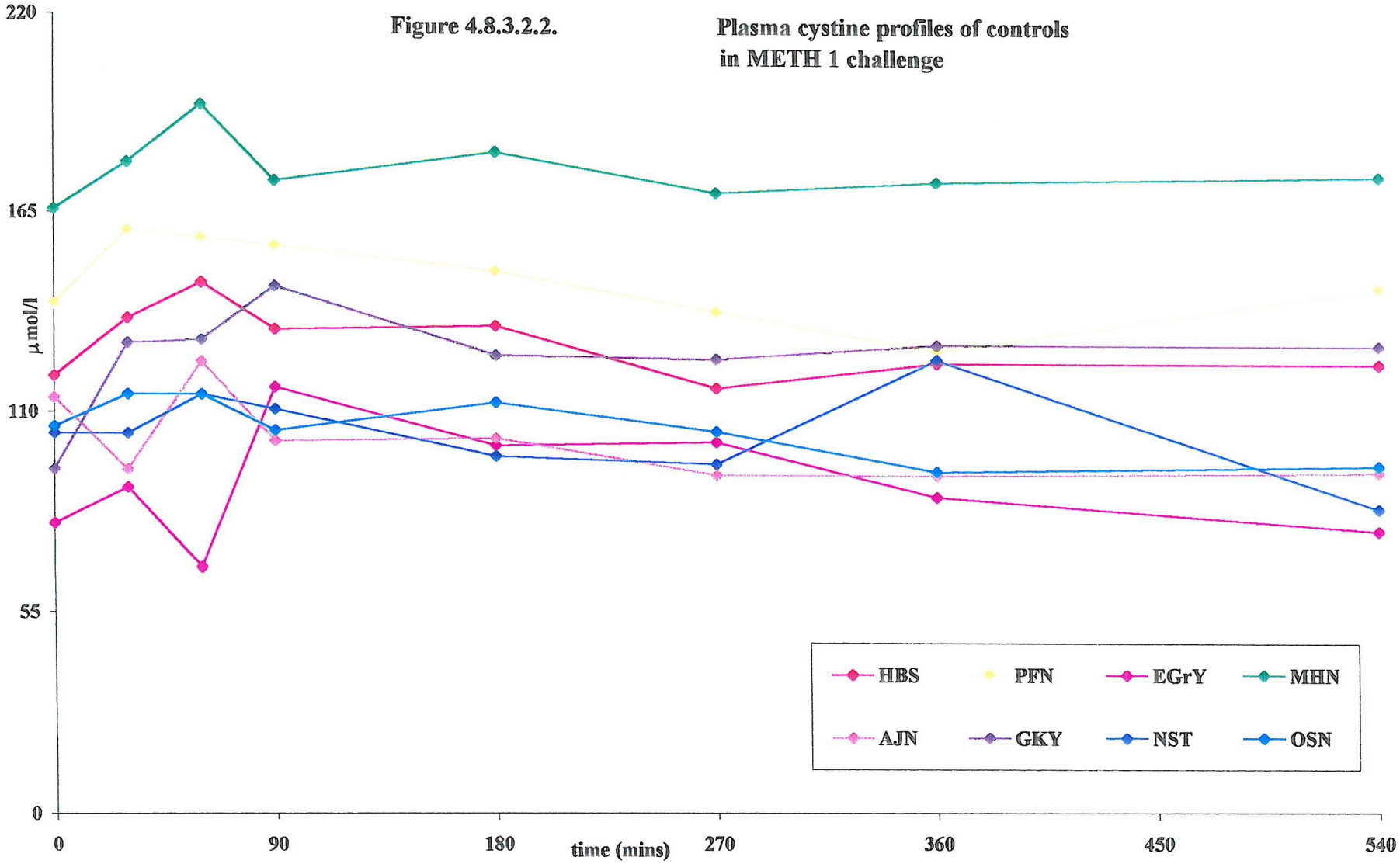


Figure 4.8.3.2.3. **Plasma cystine profiles of RAP in METH1 challenge**

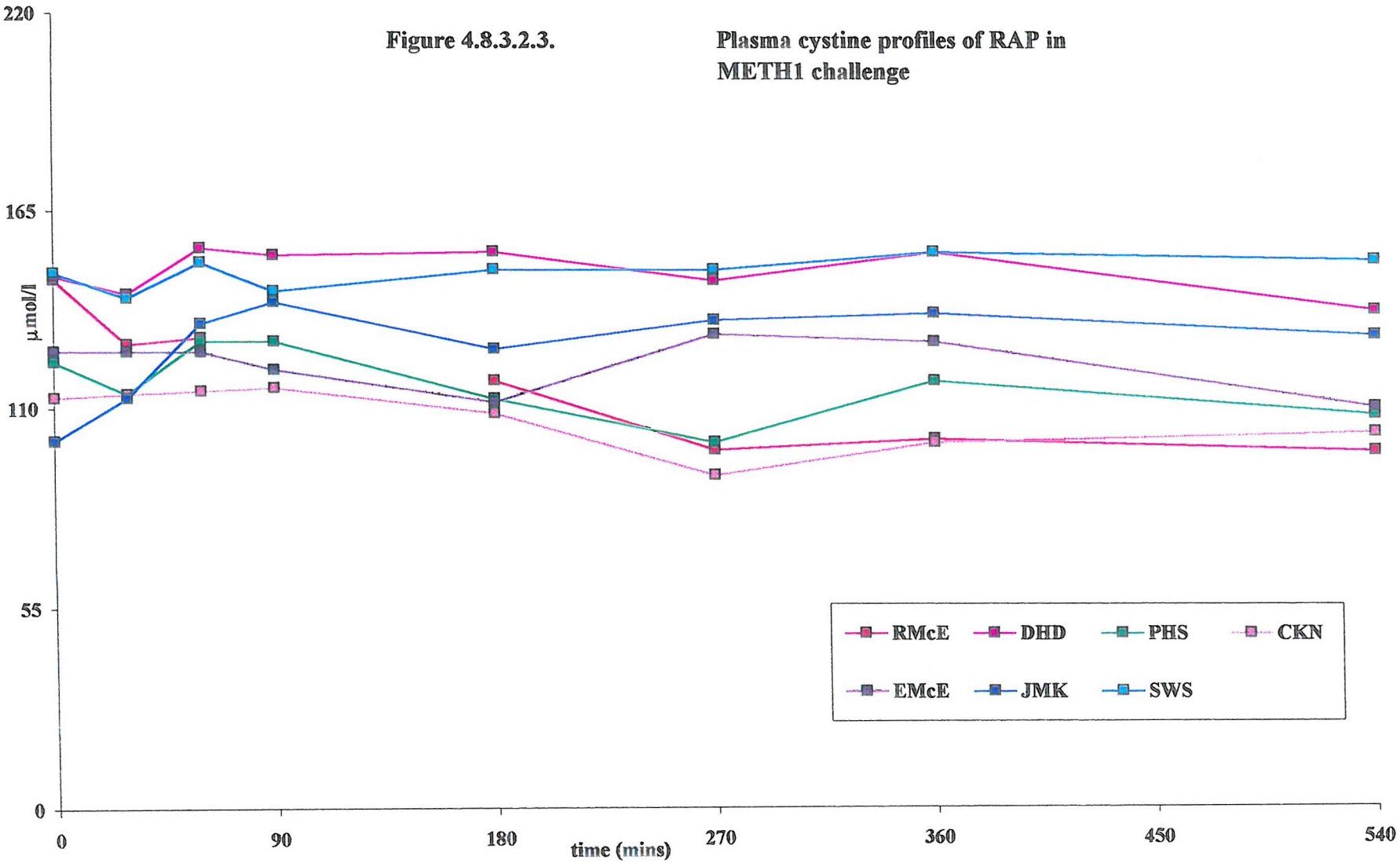


Figure 4.8.3.2.4. **Plasma cystine profiles of CP in METH1 challenge**

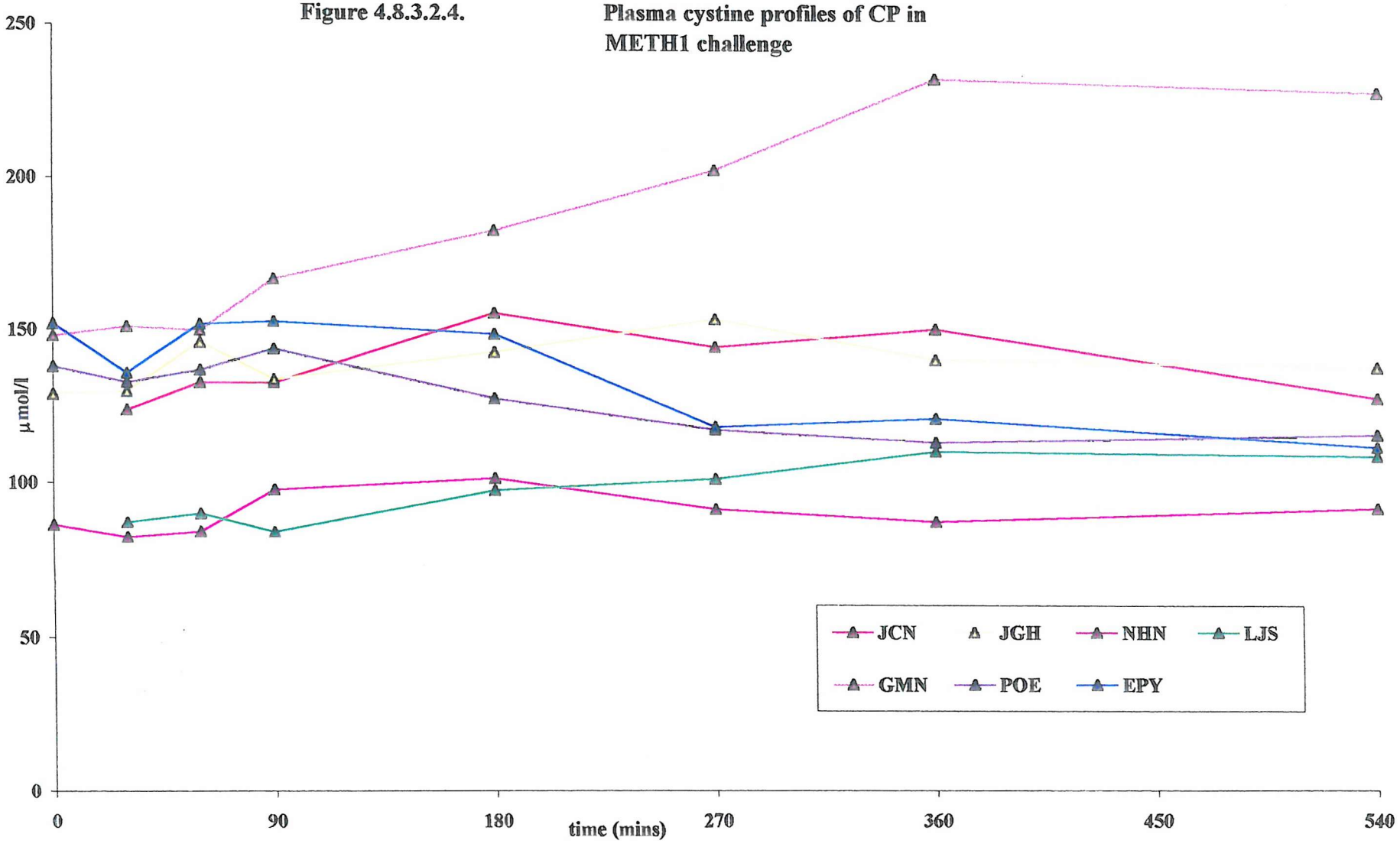


Figure 4.8.3.2.5. **Group mean (+/- 1s.d.) plasma cystine profiles: METH1 data**

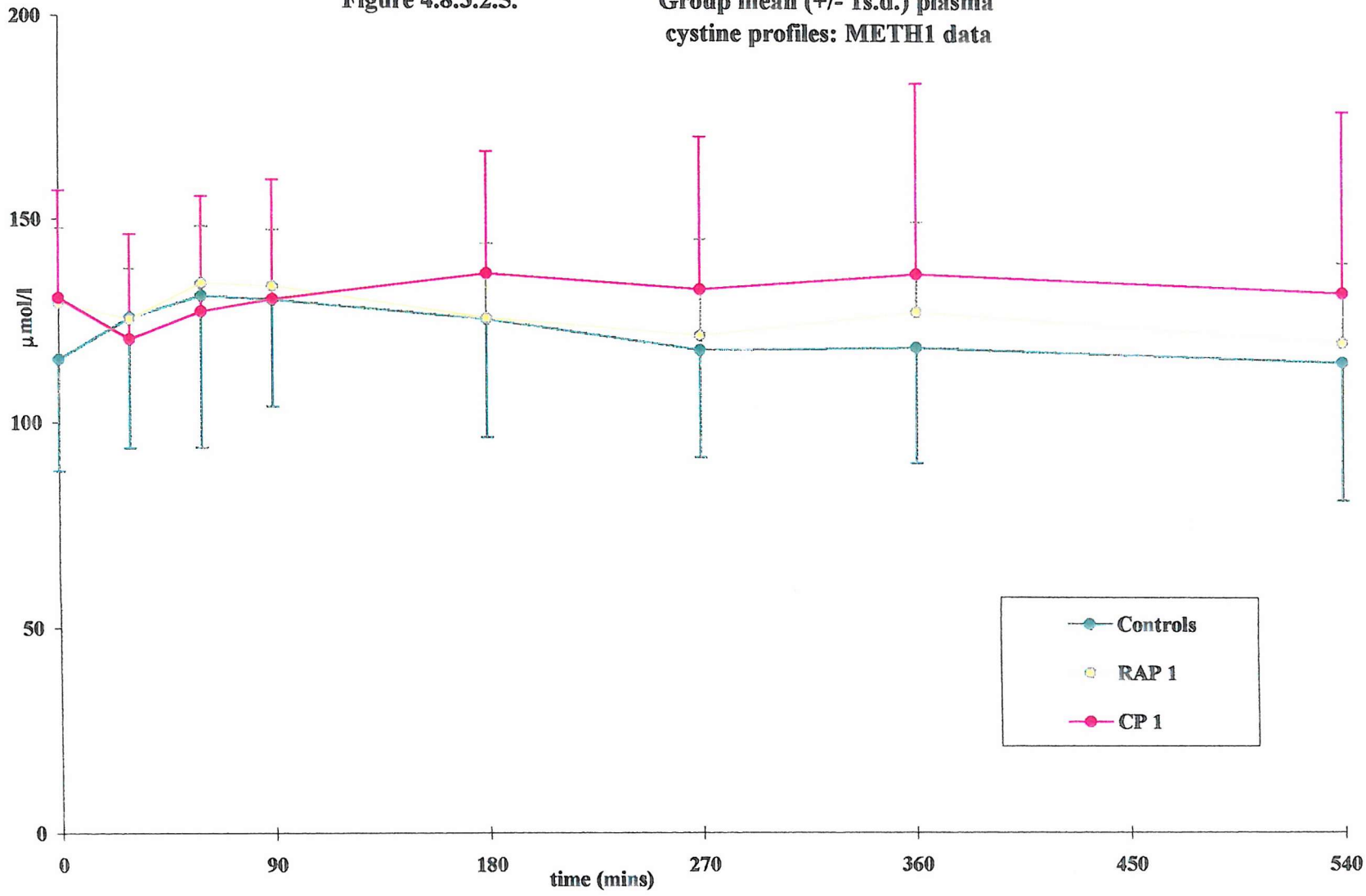


Figure 4.8.3.2.6. **Plasma cystine profiles of RAP in METH2 challenge**

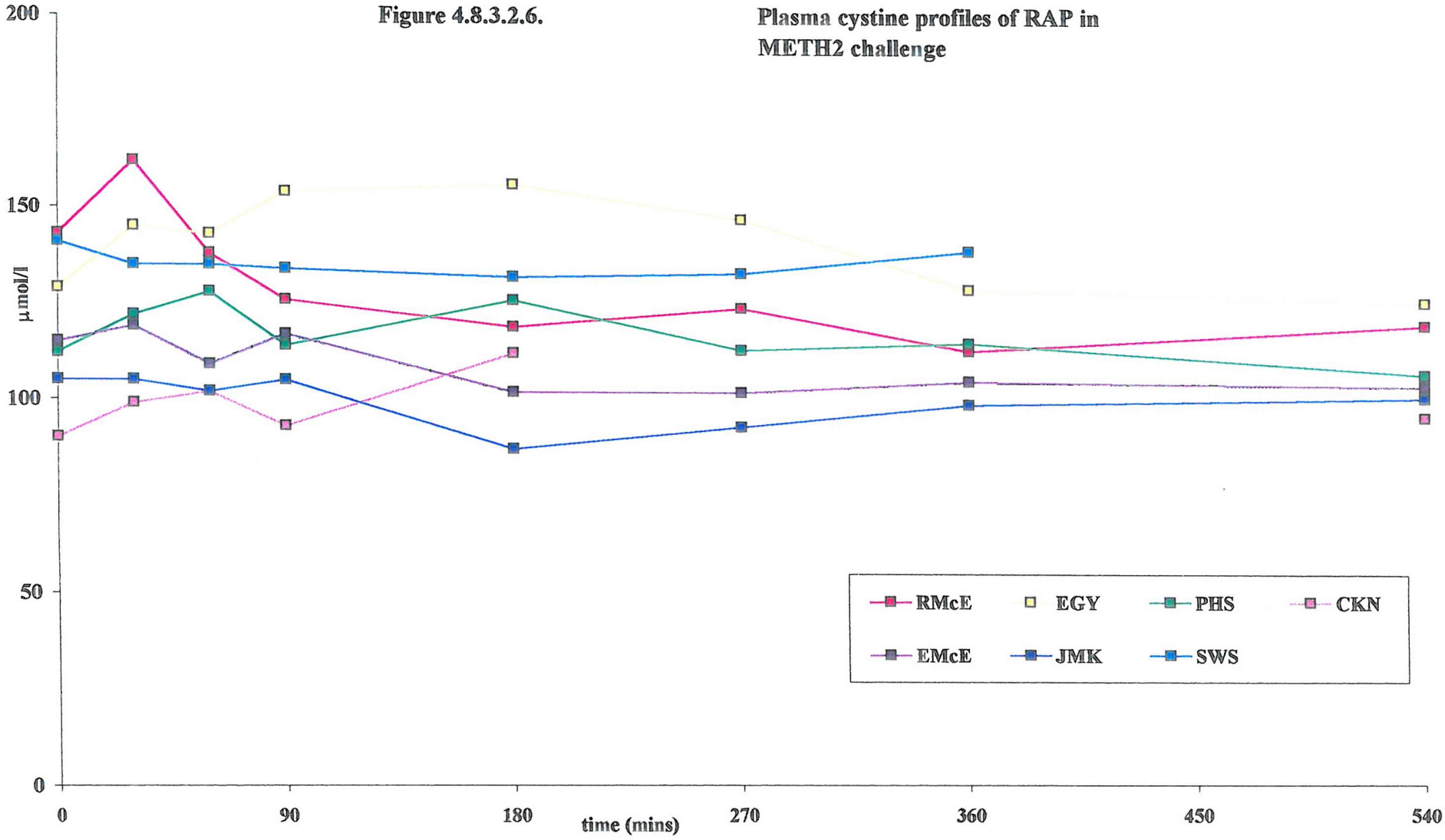


Figure 4.8.3.2.7. **Plasma cystine profiles in CP in METH2 challenge**

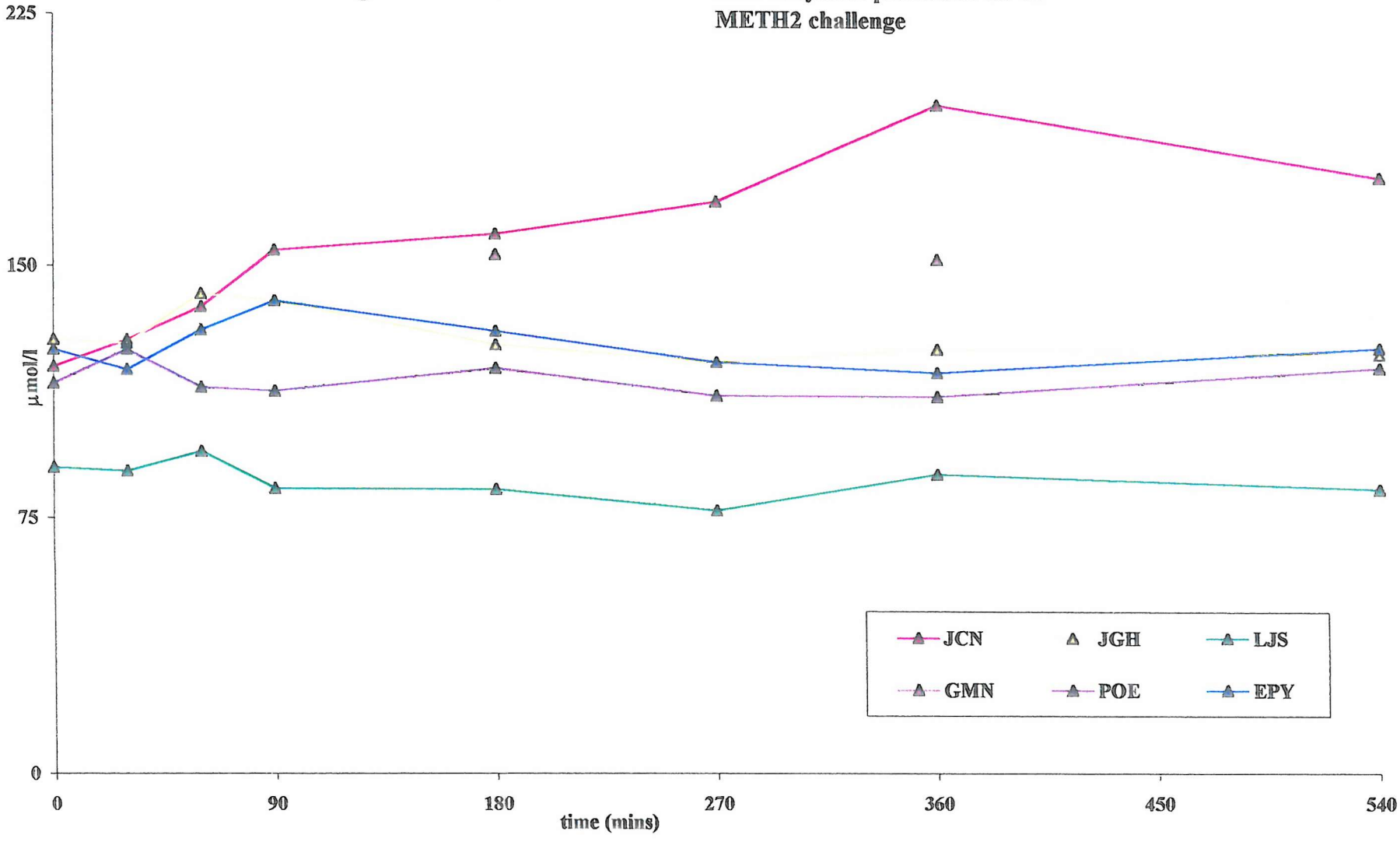
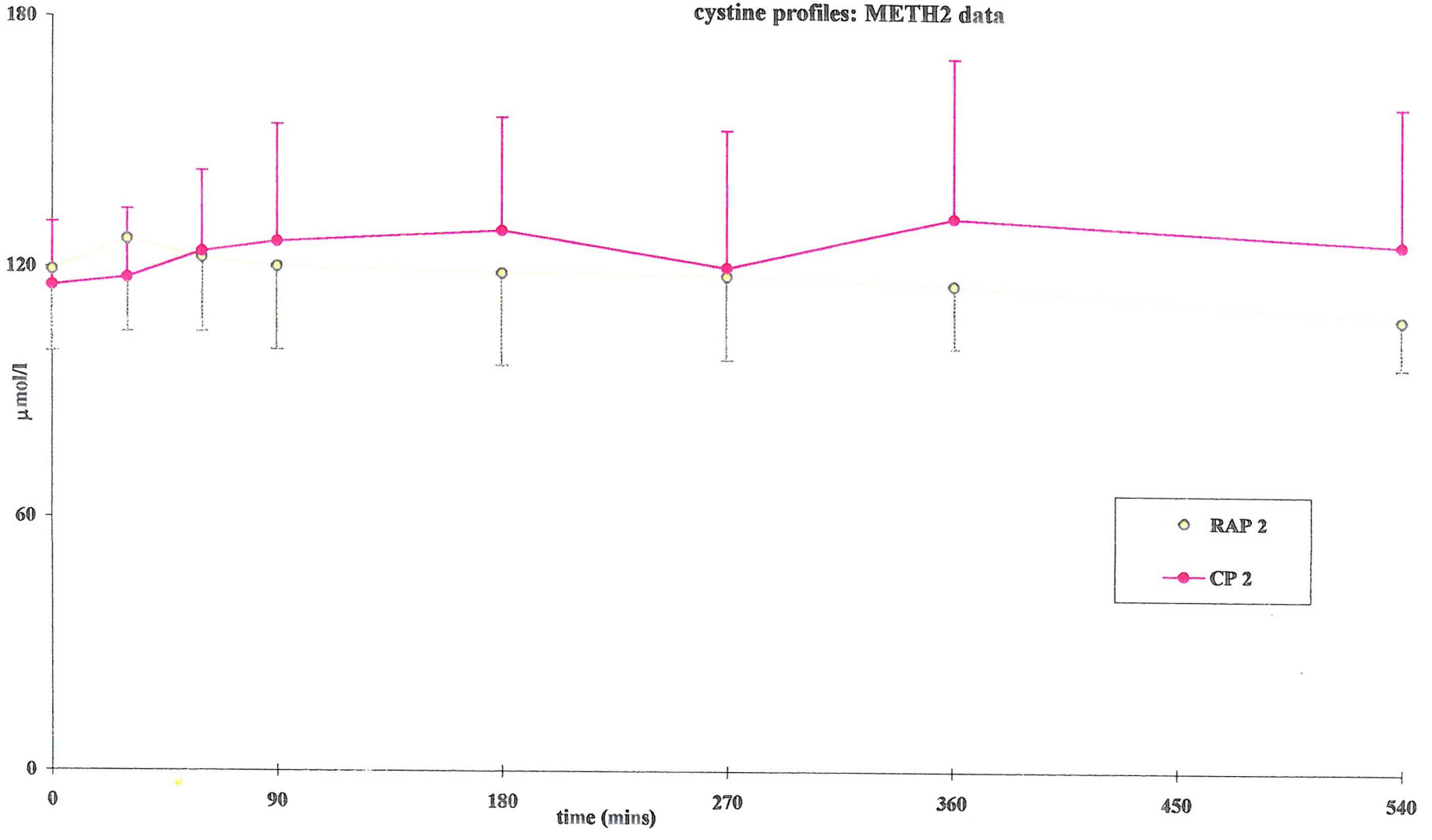


Figure 4.8.3.2.8.

Group mean (+/- 1s.d.) plasma cystine profiles: METH2 data



	Before AOT	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	METH 1	NAC 1
	Time (mins)	0	30	60	90	180	270	360	540	AUC
Control	mean	115.4	125.8	131.3	130.4	125.8	118.4	119.1	115.8	1095.6
	sd	27.2	31.8	37.2	26.1	28.9	26.2	28.2	33.9	253.2
RAP	mean	129.1	125.4	134.3	133.7	126.0	125.3	127.9	120.6	1138.3
	sd	18.5	12.5	14.1	14.1	18.4	24.2	22.1	19.9	160.4
CP	mean	130.6	120.4	127.4	130.4	137.0	133.3	137.1	132.9	1240.7
	sd	26.5	25.9	28.5	29.6	30.2	37.7	47.0	44.8	354.5
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**NAC 2**
	Time (mins)	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	**AUC**
RAP	mean	119.3	126.7	122.4	120.4	119.1	118.7	116.7	109.0	1030.0
	sd	19.4	22.2	17.7	19.9	22.2	20.0	14.9	11.6	141.9
CP	mean	115.6	117.6	124.0	126.4	129.3	120.8	133.0	127.2	1125.0
	sd	15.1	16.4	19.4	28.1	27.2	32.9	38.4	33.2	272.2

Table 4.8.3.2.3. **Group mean plasma cystine (μmol/l) and AUC (μmol/l/hr) after oral methionine load pre- and post- AOT**

Figure 4.8.3.2.9.
Plasma cystine profiles in controls in NAC 1 challenge

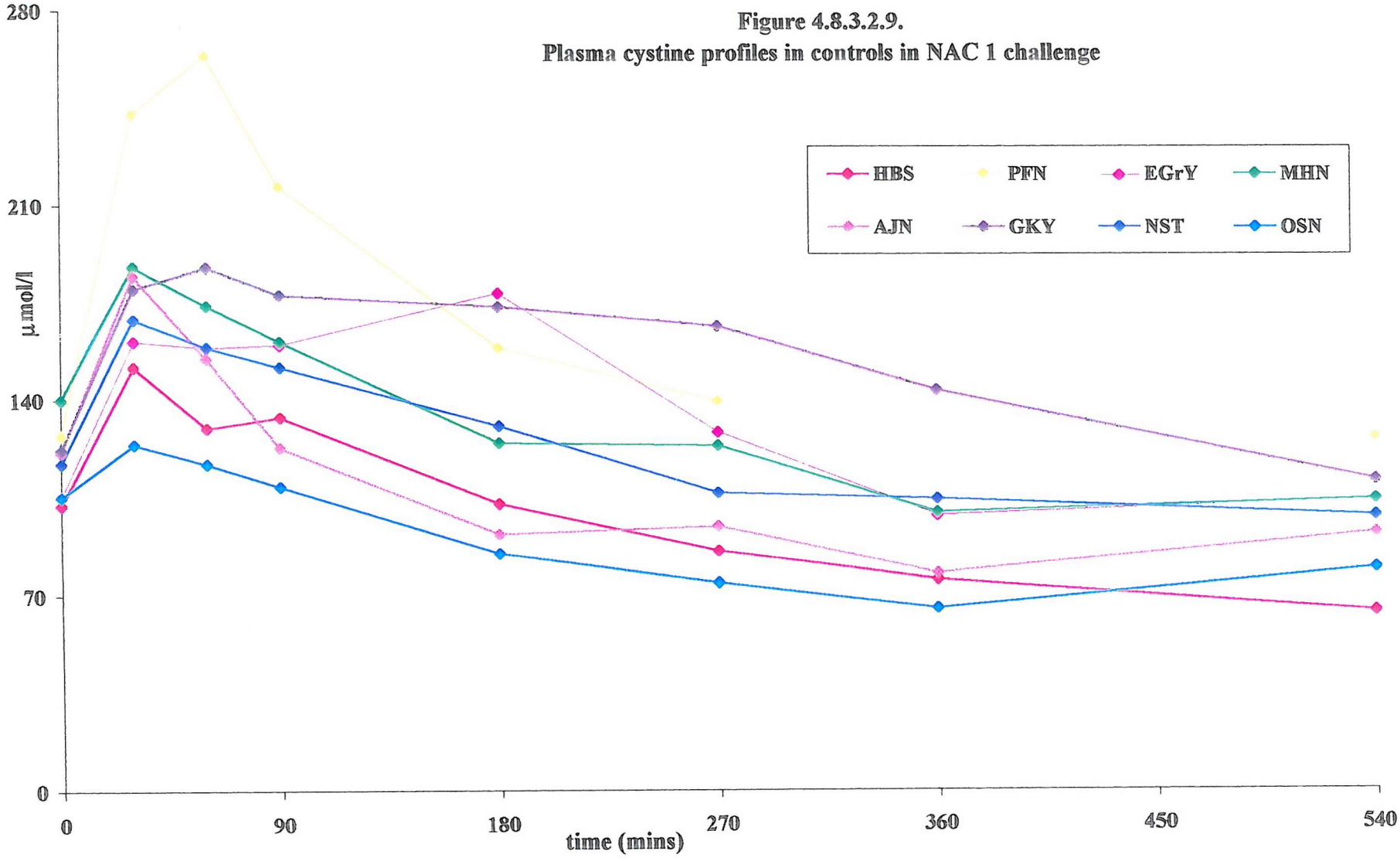


Figure 4.8.3.2.10.
Plasma cystine profiles in RAP in NAC 1 challenge

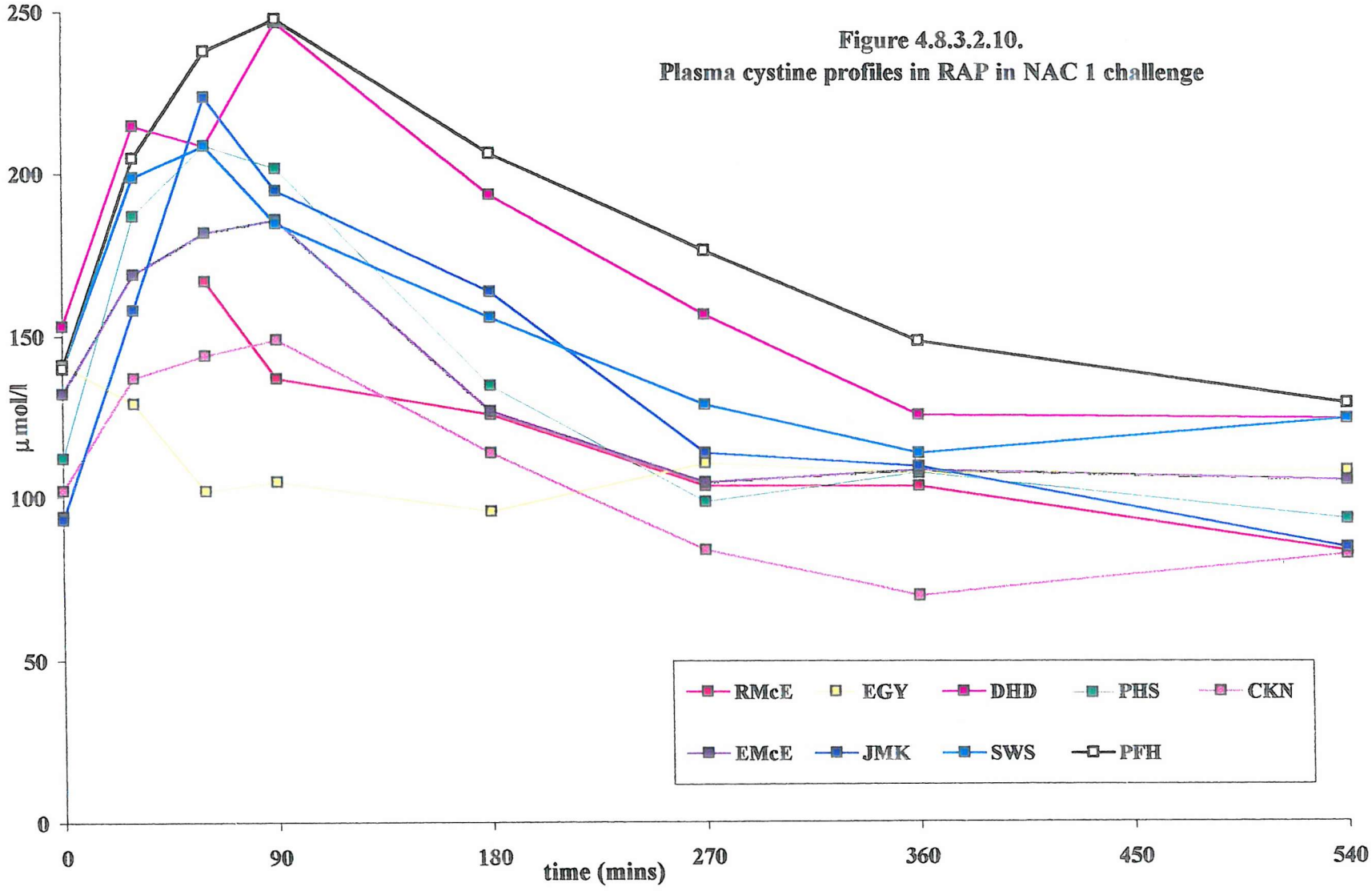


Figure 4.8.3.2.11.
Plasma cystine profiles in CP in NAC 1 challenge

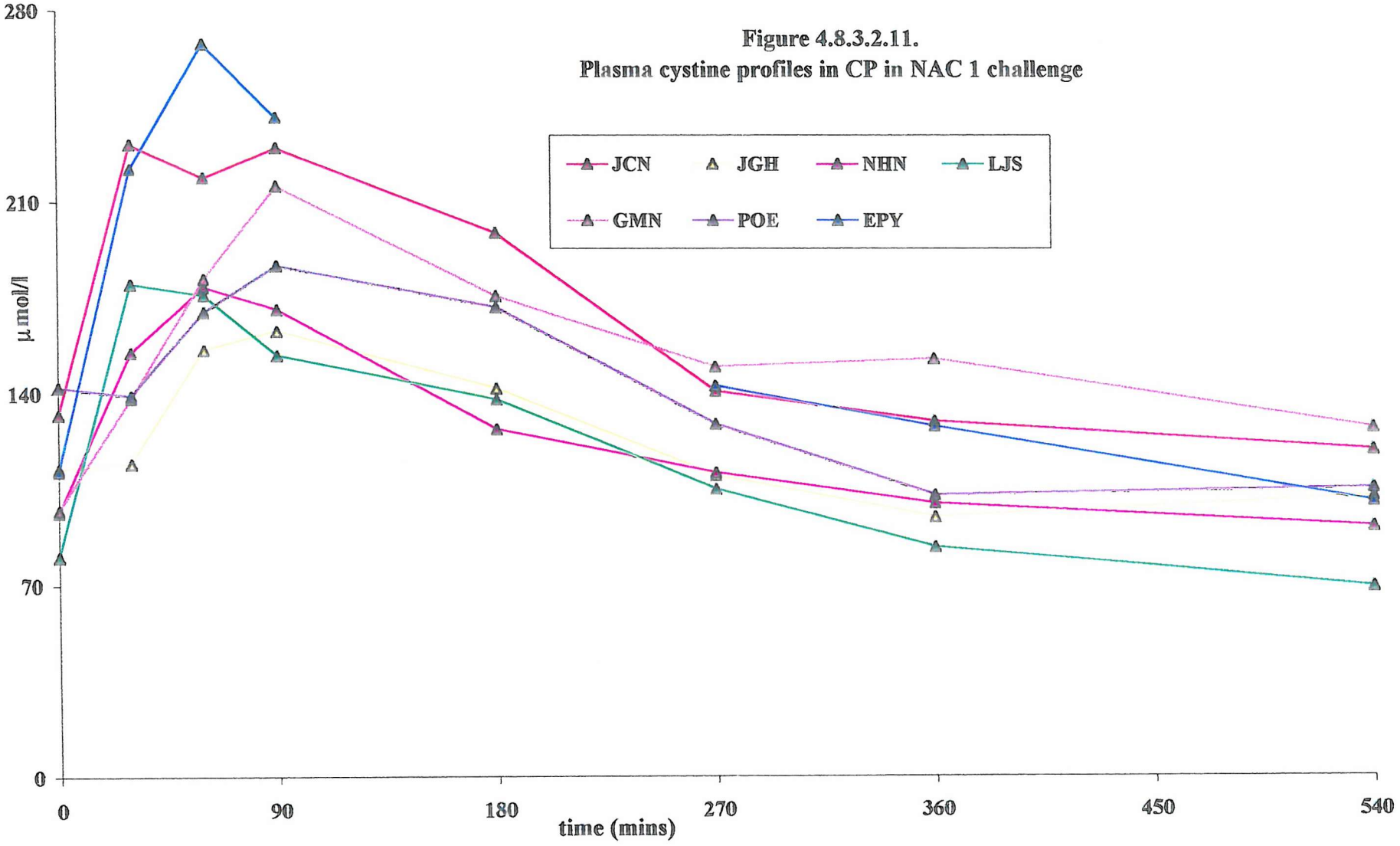


Figure 4.8.3.2.12.
Group mean (+/- 1s.d.) plasma cystine profiles: NAC1 data

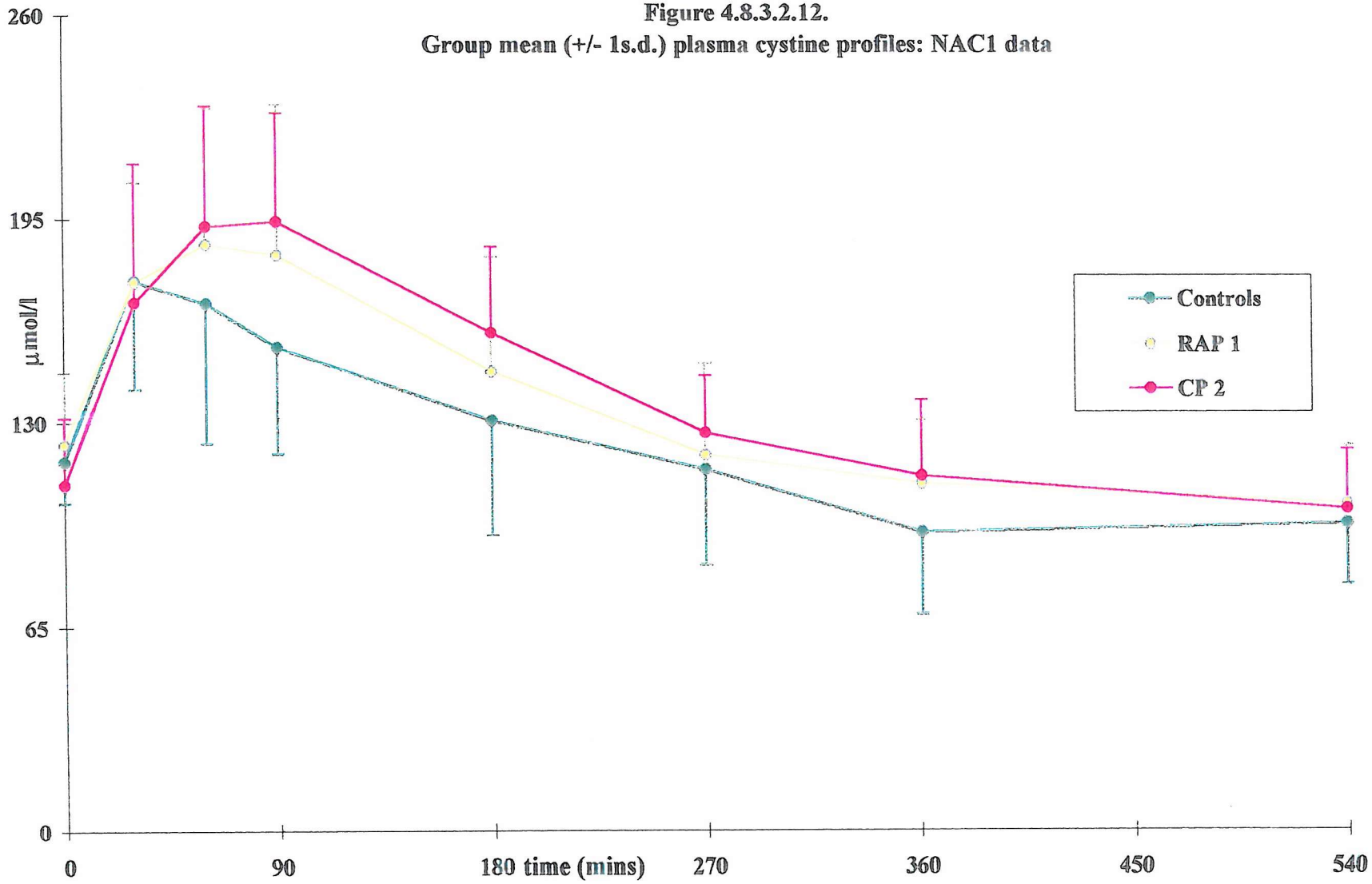


Figure 4.8.3.2.13.
Plasma cystine profiles of RAP in NAC2 challenge

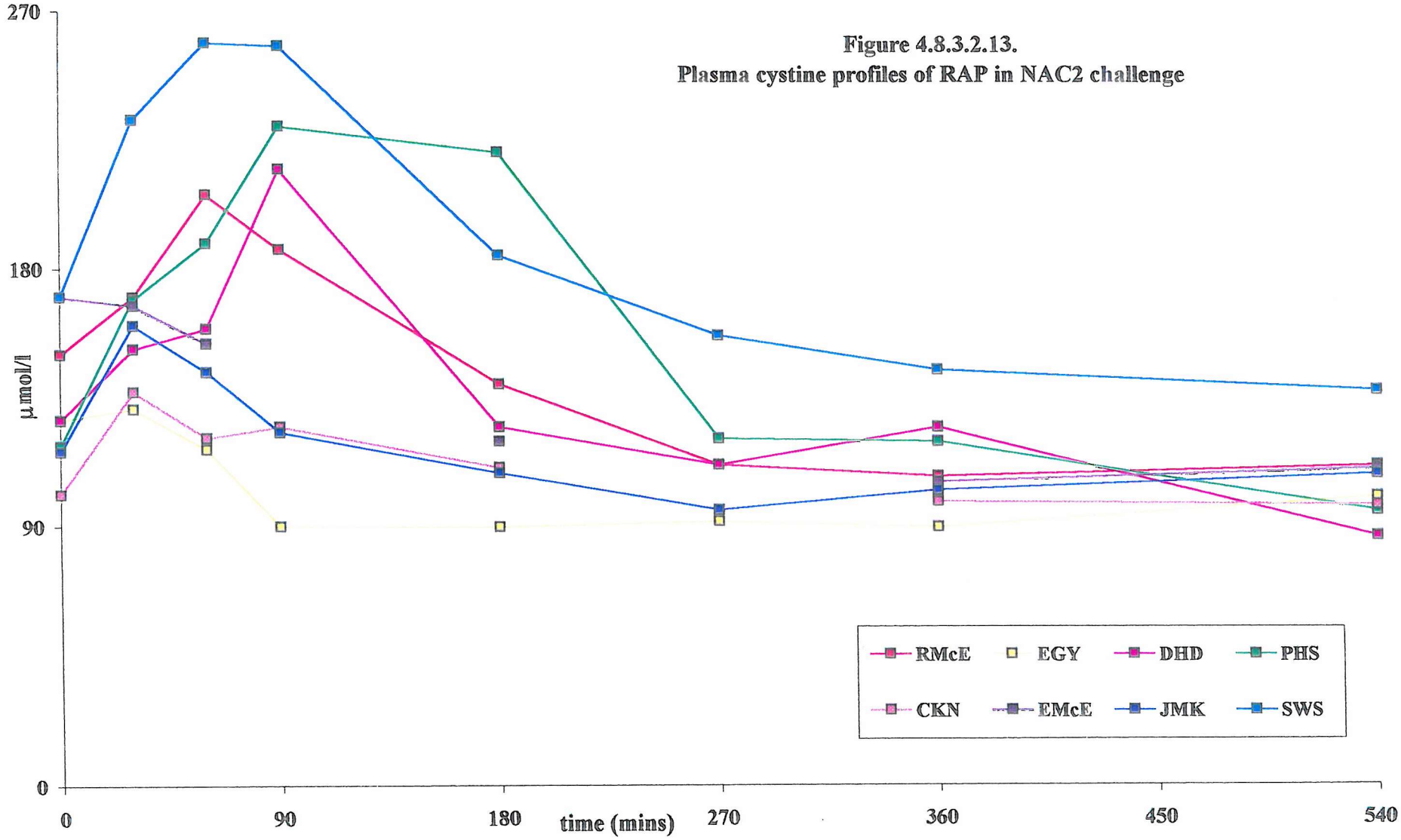


Figure 4.8.3.2.14.
Plasma cystine profiles in CP in NAC2 challenge

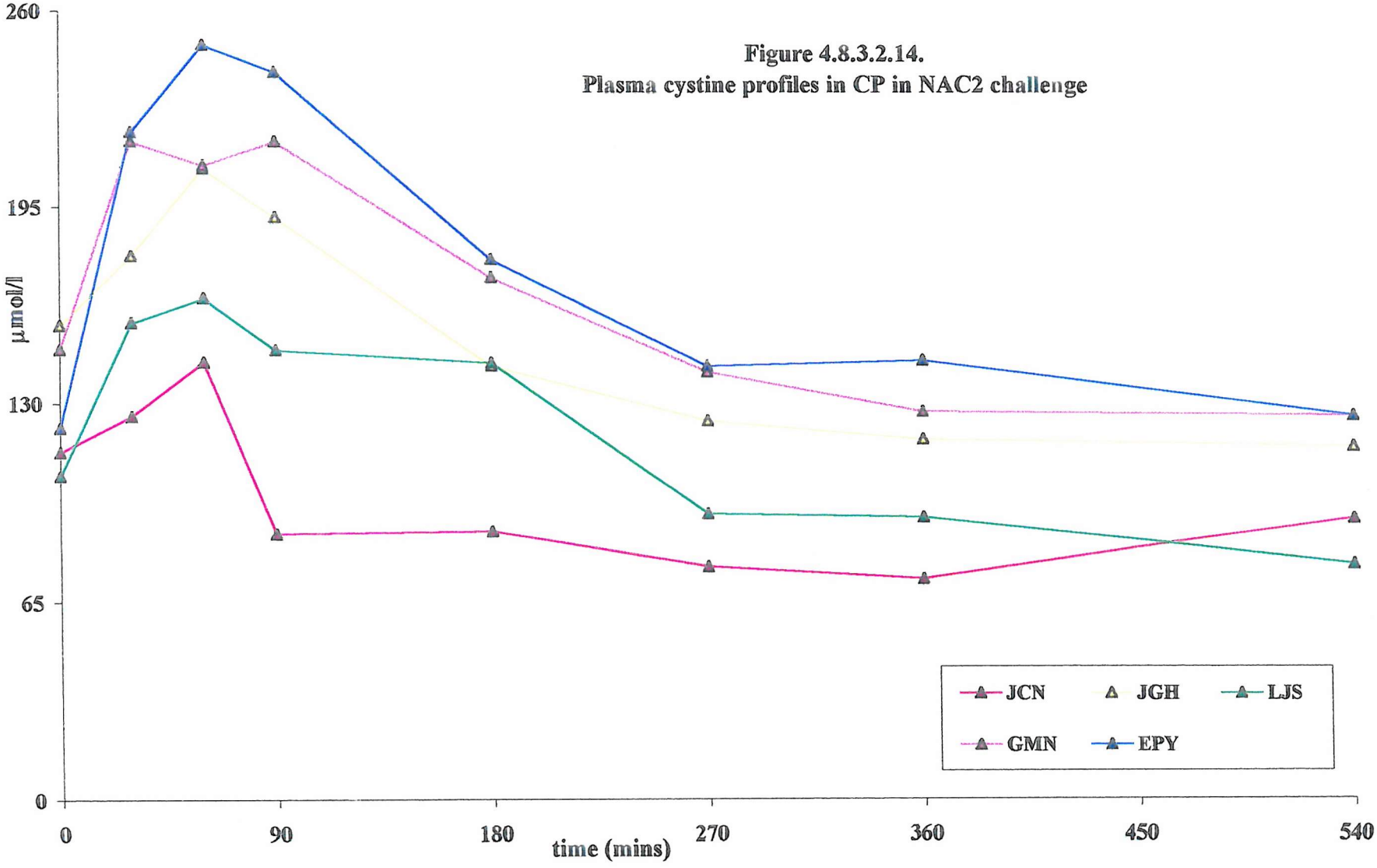
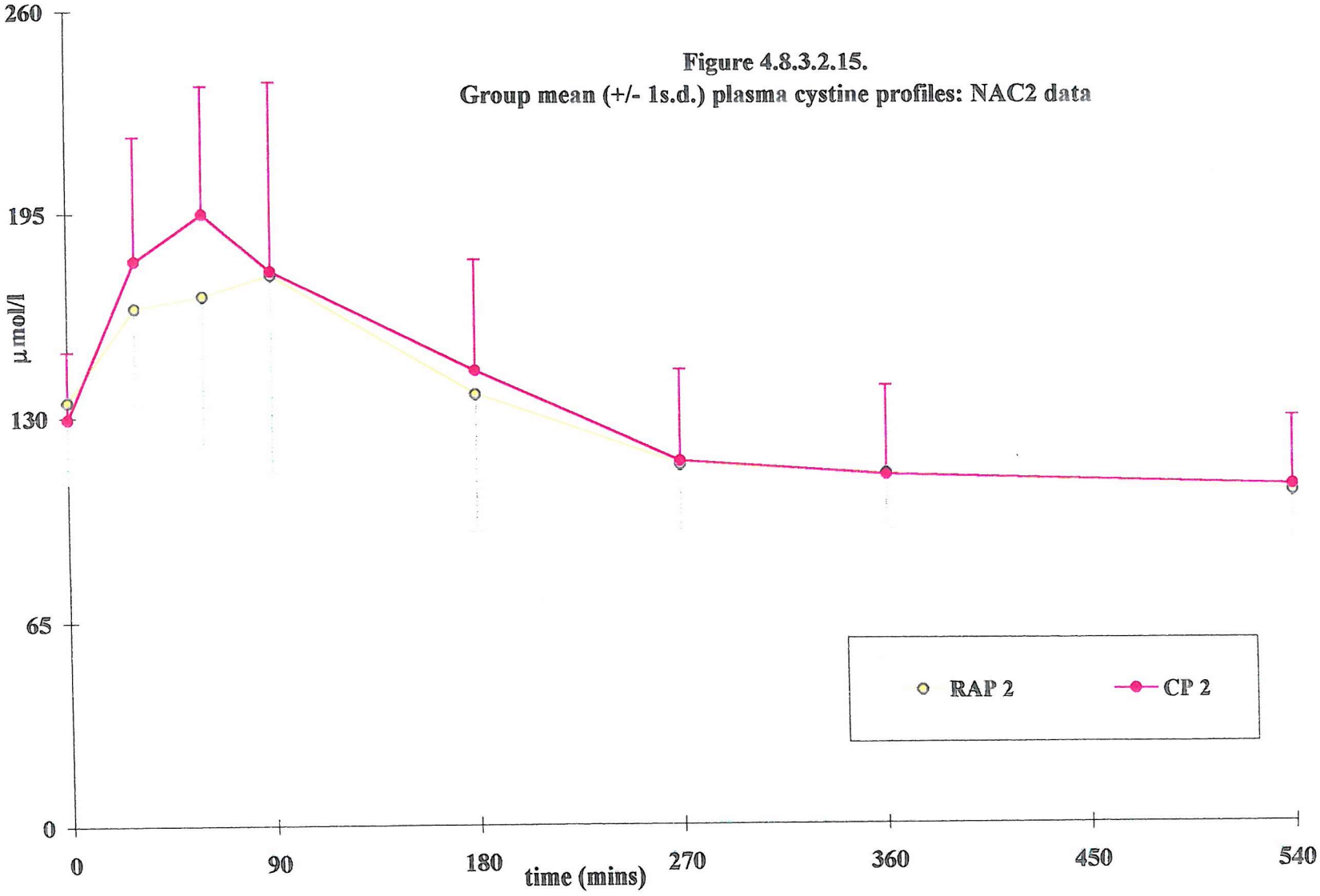


Figure 4.8.3.2.15.
Group mean (+/- 1s.d.) plasma cystine profiles: NAC2 data

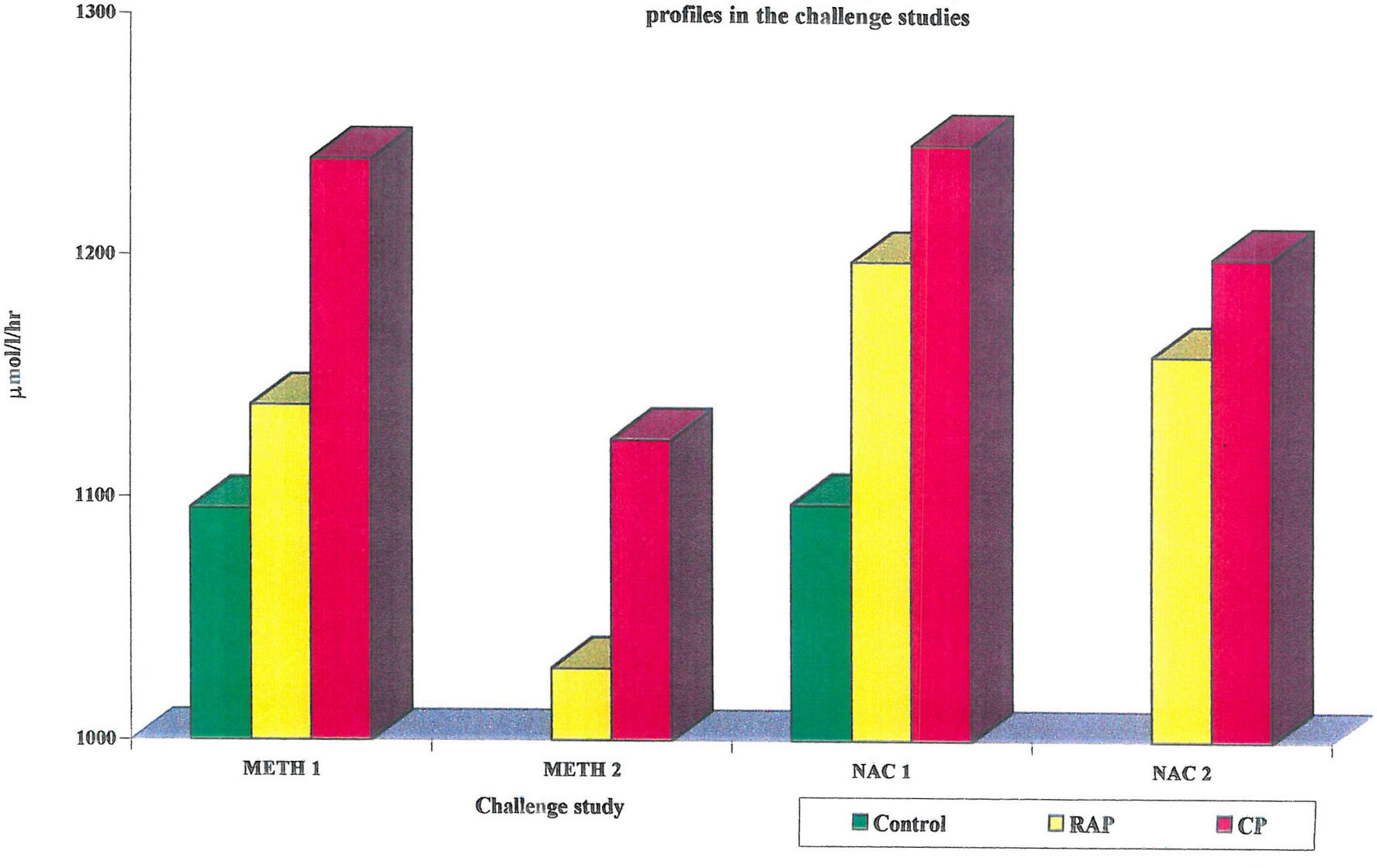


	Before AOT	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1	NAC 1
	Time (mins)	0	30	60	90	180	270	360	540	AUC
Control	mean	117.4	175.3	168.3	154.3	131.0	115.4	95.4	98.3	1098.3
	sd	13.0	34.4	44.8	33.8	36.6	30.5	25.9	19.2	246
RAP	mean	123.0	174.9	187.1	183.8	146.6	120.0	110.9	104.6	1199.2
	sd	22.9	31.9	43.2	47.7	36.8	29.7	20.7	19.0	236.8
CP	mean	110.0	168.4	192.9	194.6[a]	159.0	127.0	113.3	102.9	1248
	sd	21.5	44.4	38.3	34.5	27.6	18.3	24.3	18.9	199.9
	After AOT	NAC 2	NAC 2	NAC 2	NAC 2	NAC 2	NAC 2	NAC 2	NAC 2	NAC 2
	Time (mins)	0	30	60	90	180	270	360	540	AUC
RAP	mean	134.9	164.8	168.6	175.4	137.6	115.0	112.0	106.5	1160.5
	sd	25.7	30.9	47.5	63.4	43.9	23.3	17.4	15.3	231.5
CP	mean	129.2	179.8	195.0	176.8	145.0	116.0	111.6	108.4	1201.7
	sd	21.8	40.0	41.2	60.7	35.6	29.3	28.6	21.9	289.2

[a] $p < 0.05$ with respect to control group at same time point (2-tailed Students t-test)

Table 4.8.3.2.4. **Group mean plasma cystine concentrations (μmol/l) and AUC after oral NAC load before and after AOT**

Figure 4.8.3.2.16. **Group mean AUC for plasma cystine profiles in the challenge studies**



Group	Controls	RAP 1	RAP 2	CP 1	CP 2
period extrapolated (mins)	90-360	90-360	90-360	90-360	90-360
1	331.5	612.4	333.6	303.4	945.4
2				332.7	397.4
3	352.9	279.6	359.2	357.7	
4	434.2	285.0	244.2	298.2	342.7
5	467.5	242.6	834.0	522.3	350.0
6	921.5	347.8		300.4	
7	475.4	299.7	946.1	286.1	360.5
8	382.7	379.8	329.6		
9		370.2			
mean	*480.8*	*352.2*	*318.1*	*343.0*	*479.2*
s.d.	*202.0*	*115.5*	*322.4*	*82.8*	*261.5*
n	8	8	6	7	5
SQRT n	2.83	2.83	2.45	2.65	2.24
sem	71.42	40.83	131.62	31.28	116.92

allpancs 1	allpanc 2
612.4	243.4
279.6	191.3
285.0	194.3
242.6	525.2
347.8	503.4
299.7	251.2
379.8	313.7
370.2	277.1
303.4	287.6
332.7	338.3
357.7	251.5
298.2	
522.3	
300.4	
286.1	
allpanc mean	347.9	307.0
s.d.	100.3	111.7

Patient no. / initials

No.	Control	RAP	CP
1	HBS	RMcE	JCN
2	PFN	EGY	JGH
3	EGrY	DHD	NHN
4	MHN	PHS	LJS
5	AJN	CKN	GMN
6	GKY	EMcE	POE
7	NST	JMK	EPY
8	OSN	SWS	
9		PFH	

t-tests	half life	plasma	cystine
c 1	v	rap 1	0.17
c 1	v	cp 1	0.13
rap 1	v	cp 1	0.86
rap 1	v	rap 2	0.26
cp 1	v	cp 2	0.39
c 1	v	rap 2	0.72
c 1	v	cp 2	0.99
c 1	v	allpanc 1	0.15
allpanc 1	v	allpanc 2	0.39

Table 4.8.3.2.5. **Individual and mean group plasma cystine half-lives (mins) after NAC challenges**

4.8.3.3. Plasma Taurine

Fasting baseline plasma taurine concentrations were similar to reported values, with the exception of those reported by Armstrong 1973, whose levels were approximately double other reports, Table 4.8.3.12.1. In general, the values in the control group, tended to be lower than those of patients with pancreatitis, Table 4.8.3.3.1 - 2., but these did not reach statistical significance. Furthermore, no difference was identified in the baseline taurine level in phase 2, whether the individual had taken methionine or NAC two weeks earlier. It was thus valid to average the baseline results of phases 1 and 2, and of phases3 and 4, of each group, before and after AOT. These combined results also failed to reveal any difference between the control and patients groups, although prior to Bonferroni the combined patient group after AOT were higher than controls, Table 4.8.3.3.2. (shaded areas). No difference in plasma taurine concentration was found between genders within each group at baseline. AOT did not alter any of these observations.

Figures 4.8.3.3.1. - 3. plot the mean plasma taurine profiles of the three subject groups after oral loading with NAC and methionine. Figures 4.8.3.3.4. - 5. collate these profiles of the subject groups into before and after AOT values. Comparison between mean levels in the three groups throughout the profile period after oral loading with either NAC or methionine revealed only one difference; higher plasma taurine in patients with CP compared to RAP at the 360 minute time-point after AOT. This difference remains after Bonferroni (16 comparisons), Figure 4.8.3.3.5 (***asterisked***) and Table 4.8.3.3.1.

Figure 4.8.3.3.6. shows the mean AUC for taurine in these challenge studies, grouped by disease state and type of challenge. Patients with pancreatitis had a trend towards greater plasma taurine areas, which failed to reach significance, even for the AUC observed after patients with chronic pancreatitis, treated for 10 weeks with AOT, were loaded with NAC, is compared to the AUC of controls subjects ($p = 0.03$ prior to Bonferroni correction, ***asterisked***).

Similarly, combining the results for the AUC of the taurine profiles, in all patients with pancreatic disease after AOT, no difference to the control group was observed following a methionine or NAC load, using Wilcoxon Rank Sum Test (WRST) non-parametric analysis, Table 4.8.3.3.3.

Summary of plasma taurine results in challenge study

- Plasma taurine levels were similar in this study to those previously reported.
- The mean baseline concentration in the control group was no different to patients with a history of pancreatitis.
- Oral loading with NAC and methionine had no effect on plasma taurine profiles.
- The AUC was the same in controls and patients.

	Time (mins)	0	30	60	90	180	270	360	540	AUC
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control	mean	82.0	76.0	74.1	79.0	75.5	76.5	83.5	89.4	724
	sd	17.0	22.2	22.8	18.9	27.5	26.9	26.8	33.6	166
RAP	mean	98.6	100.1	101.4	85.3	101.9	96.0	93.2	105.4	839
	sd	31.6	25.8	26.1	26.4	32.6	28.2	34.7	37.3	226
CP	mean	79.4	103.4	95.6	100.2	90.0	97.7	106.0	124.9	932
	sd	23.5	37.5	23.6	36.4	23.3	24.3	16.4	35.4	156
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP	mean	96.5	88.8	89.4	98.3	94.6	89.7	106.4	106.4	887
	sd	21.6	15.3	23.1	29.4	26.6	20.9	30.4	24.3	169
CP	mean	97.0	114.0	109.5	102.5	93.7	114.8	107.2	106.5	952
	sd	24.5	42.9	47.5	44.4	30.9	39.2	48.2	30.7	329
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control	mean	73.3	78.6	85.0	76.1	78.4	86.3	90.6	77.3	747
	sd	19.1	17.5	31.4	15.7	22.8	31.3	30.5	17.1	160
RAP	mean	100.4	81.3	84.6	103.4	99.6	91.4	98.2	101.3	871
	sd	27.8	22.7	30.0	26.2	23.9	23.2	38.5	34.7	227
CP	mean	90.0	90.6	94.7	99.1	92.1	94.4	94.4	102.9	861
	sd	30.6	33.2	20.8	22.4	17.5	24.5	14.8	18.7	131
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP	mean	97.6	93.3	105.5	101.3	93.1	87.7	92.6	81.5	824
	sd	26.8	21.1	15.3	33.4	37.9	17.8	9.5	17.6	139
CP	mean	103.8	113.8	112.0	115.8	90.0	94.5	118.2[a]	94.7	939
	sd	26.4	29.2	21.2	36.5	21.5	21.1	11.6	28.1	132

a $p = 0.001$ with respect to NAC2 RAP group (2-tailed Students t-test)

Table 4.8.3.3.1. Group mean (s.d.) of plasma Taurine (μmol/l) and AUC(μmol/l/hr) in challenge studies.

	Phase		Control	RAP	CP	Allpanc		*t tests*		
	Before AOT	**means**	76.6	99.4	85.8	94.8	c v cp	0.174	c v allp1	0.045
Both genders		**sd**	16.5	25.1	4.3	21.2	c v rap	0.052		
	After AOT	**means**		97.0	100.4	98.5	rap1 v cp1	0.175		
		sd		17.6	19.1	17.6	rap2 v cp2	0.742	c v allp2	0.010
	Before AOT	**means**	76.8	113.5	86.5	101.9	c v cp	0.329	c v allp1	0.077
Male		**sd**	18.9	28.8	4.9	25.1	c v rap	0.079		
	After AOT	**means**		102.5	105.8	103.9	rap1 v cp1	0.156		
		sd		10.0	21.5	15.0	rap2 v cp2	0.792	c v allp2	0.029
	Before AOT	**means**	76.2	85.3	83.5	84.9	c v cp	N/A	c v allp1	0.433
Female		**sd**	15.1	10.3	N/A	9.0	c v rap	0.430		
	After AOT	**means**		88.0	89.8	88.7	rap1 v cp1	N/A		
		sd		26.3	9.5	9.0	rap2 v cp2	0.923	c v allp2	0.351

t tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.960	0.143	N/A
After AOT		0.444	0.095

Table 4.8.3.3.2. **Fasting baseline plasma Taurine (μmol/l) in mixed- and single- gender groups (mean 1 s.d.)**

Figure 4.8.3.3.1. **Mean plasma taurine profiles: controls group data (means +/- 1 sd)**

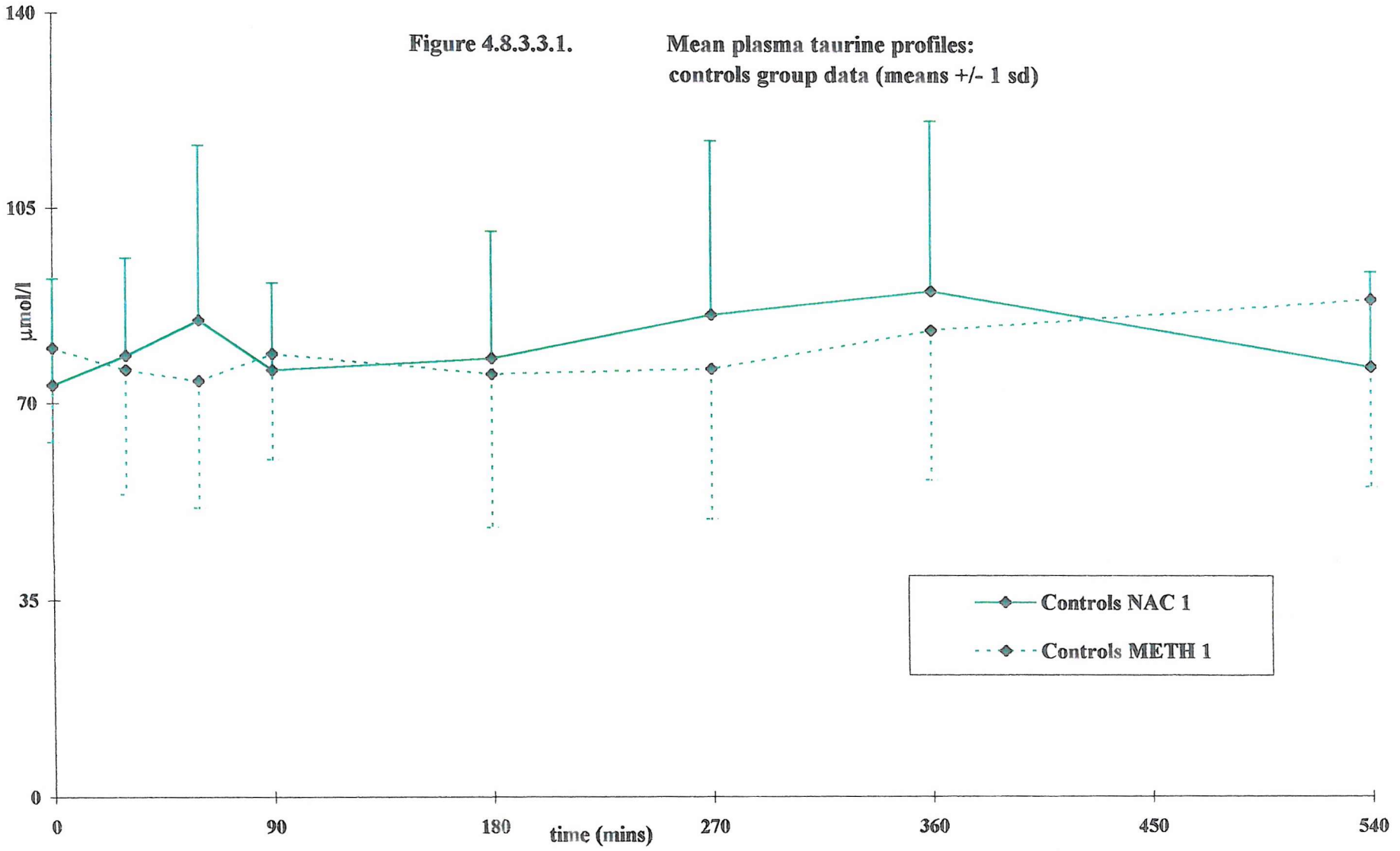


Figure 4.8.3.3.2. **Mean plasma taurine profiles: RAP group data (means +/- 1 sd)**

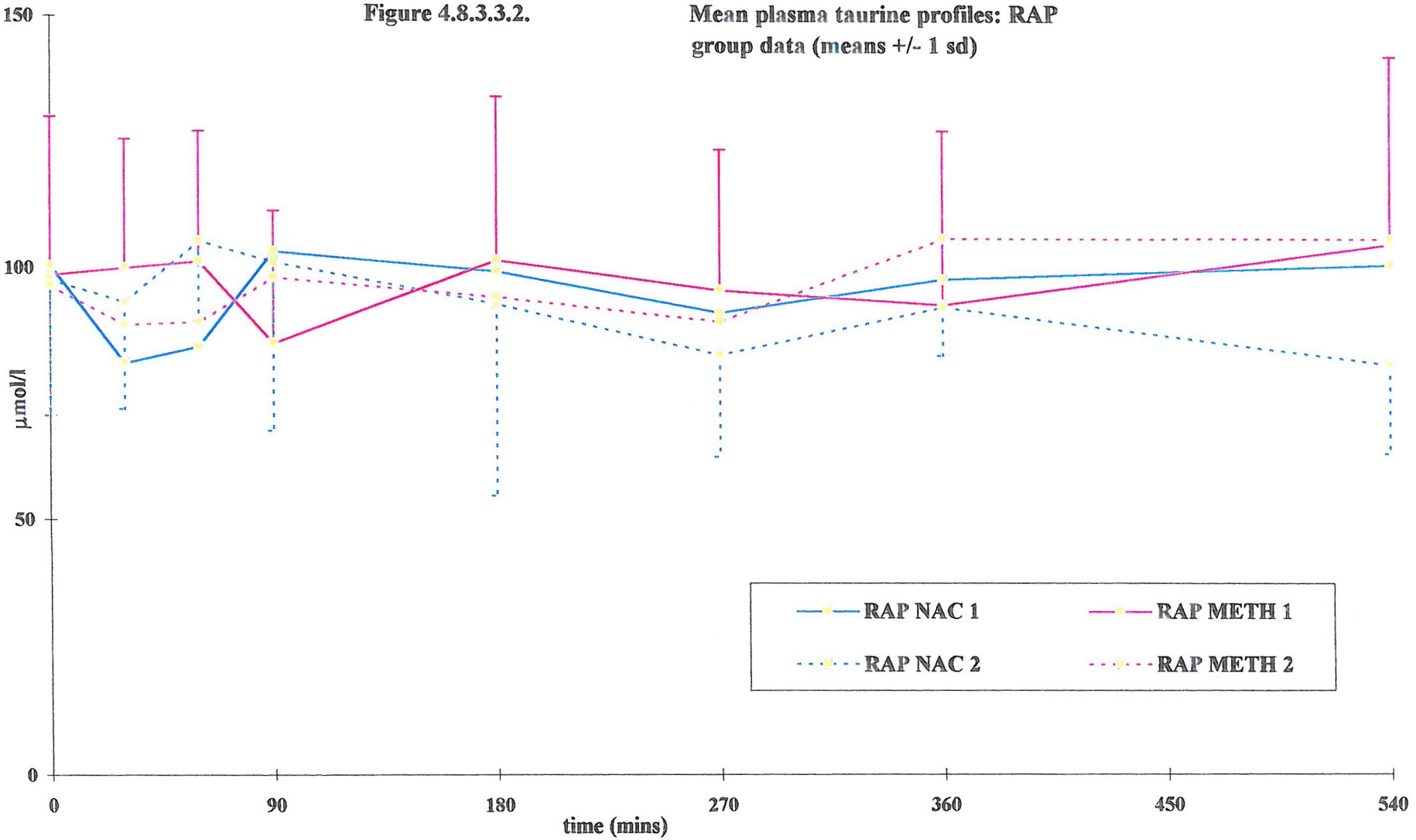


Figure 4.8.3.3.3. **Mean plasma taurine profiles: CP group data (mean +/- 1 sd)**

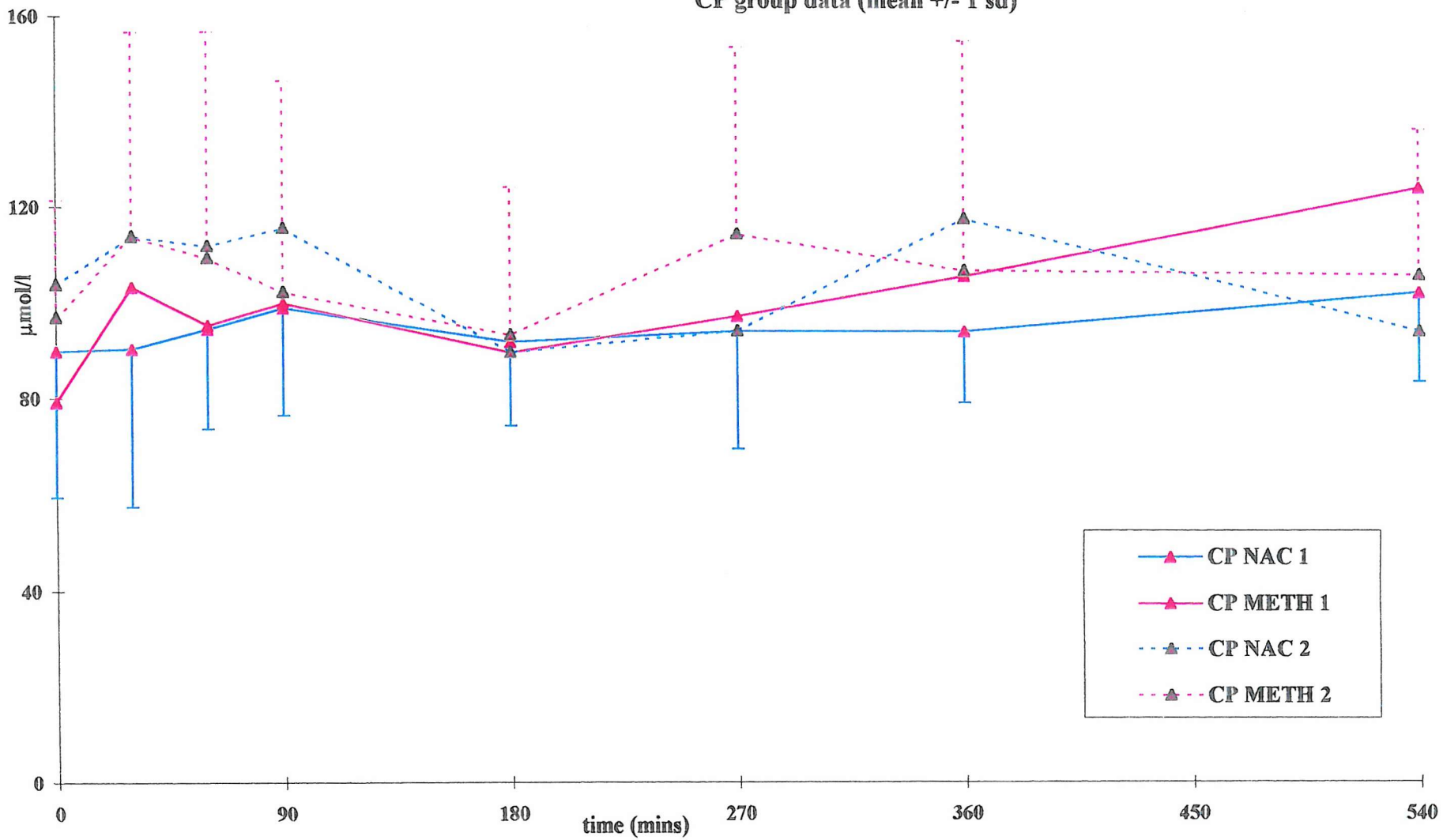


Figure 4.8.3.3.4. **Mean plasma taurine profiles: group data before AOT**

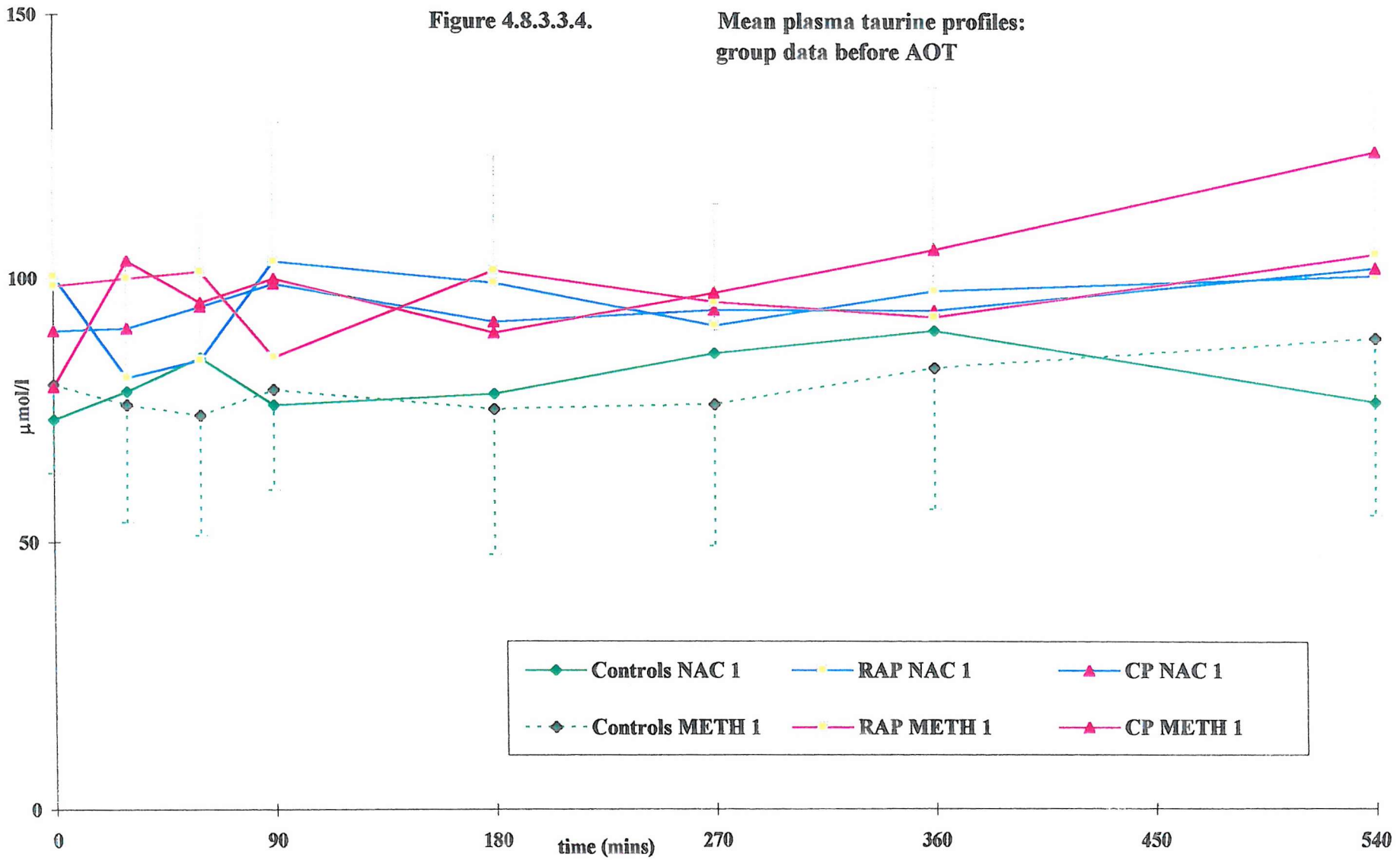


Figure 4.8.3.3.5. **Mean plasma taurine profiles: group data after AOT**

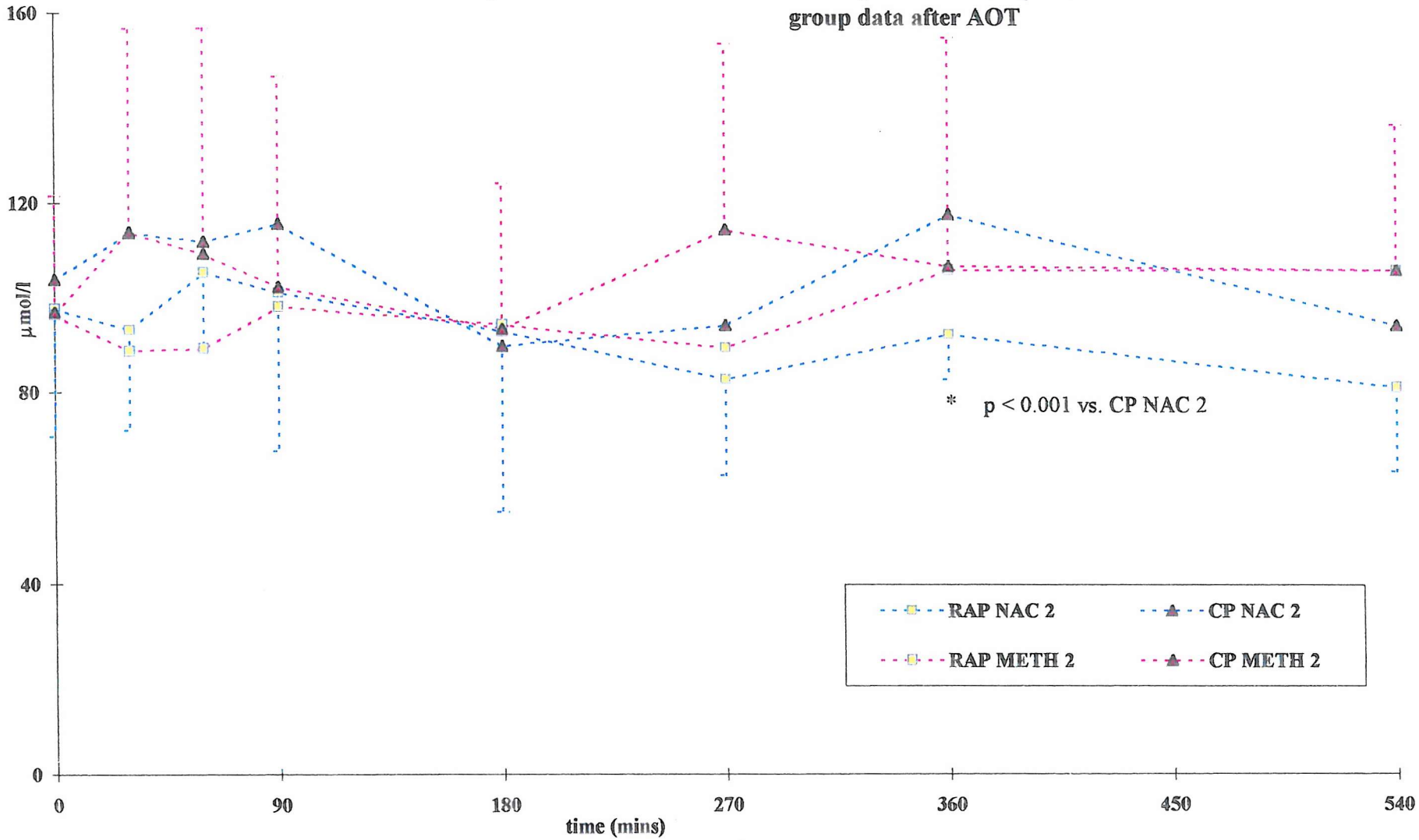
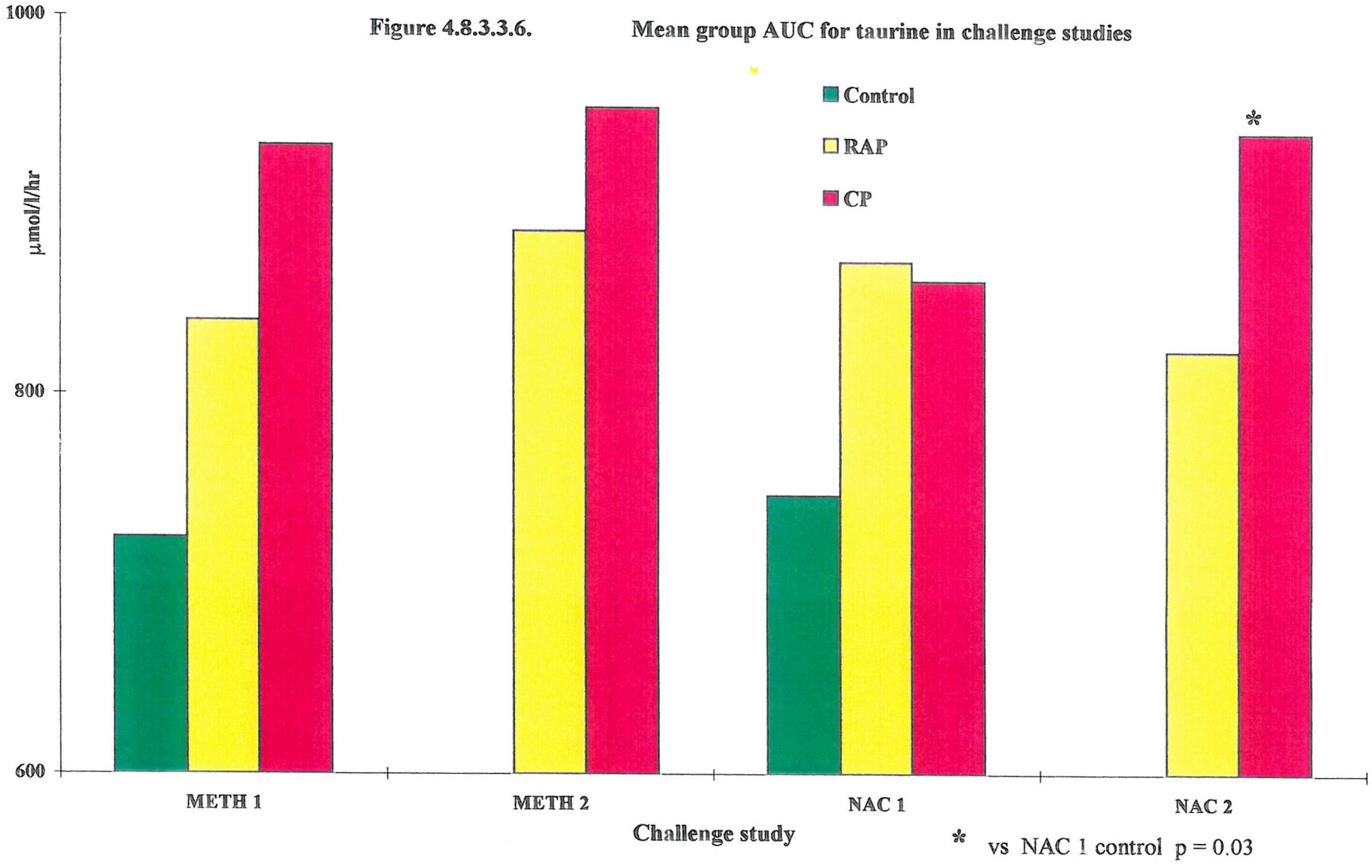


Figure 4.8.3.3.6. **Mean group AUC for taurine in challenge studies**



* vs NAC 1 control p = 0.03

	AUC	AUC	AUC	AUC
CS	**meth1**	**meth2**	**NAC1**	**NAC2**
AA	**taurine**	**taurine**	**taurine**	**taurine**
group	**controls**	**allpanc**	**controls**	**allpanc**
	684.0	851.5	559.8	855.3
	757.3	693.3	926.8	808.8
	359.5	1002.2	513.5	678.8
	707.5	856.3	838.0	1022.0
	918.0	645.5	763.5	595.3
	728.5	1124.3	785.5	922.5
	854.0	860.0	943.3	786.0
	783.0	1062.8	649.5	920.8
		1577.0		943.5
		816.8		1113.8
		704.8		1038.5
		879.5		850.0
		707.5		947.0
		1027.3		743.3
WRST	M1 v M2	**NS**	NAC1 v NAC2	**NS**

CS - Challenge study
AA - amino acid
WRST - Wilcoxon Rank Sum Test
NS - not significant

Table 4.8.3.3.3. **Individual AUC for plasma taurine (μmol/l/hr) after methionine load post-AOT vs. controls.**

4.8.3.4. Plasma Valine

Fasting baseline plasma valine concentrations were within the reported normal range, Table 4.8.3.12.1. Patients with chronic pancreatitis had significantly lower fasting plasma valine 186.5 +/- 28.5 μmol/l (mean +/- 1 s.d.) than subjects with recurrent acute pancreatitis 252.2 +/- 38.4 μmol/l ($p < 0.002$). Comparison with the control 246.1 +/- 51.5 μmol/l group did not reach significance, although when genders were separated, males with CP 195.3 +/- 21.9 μmol/l had a lower plasma valine than healthy males 272.1 +/- 14.5 μmol/l ($p < 0.0004$), Table 4.8.3.4.1.

After 10 weeks AOT, the mean fasting plasma valine in patients with chronic pancreatitis was 195.3 +/- 21.9 and in recurrent acute pancreatitis 244.7 +/- 52.6 μmol/l (NS. $p = 0.04$, Students t-test). Table 4.8.3.4.2. reports the group mean plasma valine concentration profiles in the various challenge studies. Statistical comparison of these results, Table 4.8.3.4.3. reveals that the initial low plasma valine concentration in the profile of patients with CP compared to RAP following oral methionine is not observed with NAC, or after AOT.

Figures 4.8.3.4.1. - 3. plot the profiles of plasma valine observed after oral loading with NAC and methionine in the three subject groups. Figures 4.8.4.3.4. - 5. collate the profiles of the three subject groups by challenge compound. These figures reveal that oral loading with either NAC or methionine tended to resulted in a fall of plasma valine concentration usually reaching a nadir by 3 hours, before returning to pre-loading levels. The statistical analyses of these appear in Table 4.8.3.4.4.

The mean AUC for the challenge studies appear in Table 4.8.3.4.2. and are plotted in Figure 4.8.3.4.6. Patients with CP had consistently lower plasma valine concentrations than controls and patients with RAP. This reached significance compared to the RAP group, before AOT, following an oral load of methionine, ($p < 0.001$, *asterisked*), Table 4.8.4.3.5. The AUC following an oral load of NAC, in patients with chronic pancreatitis before *and* after 10 weeks AOT compared to controls subjects or RAP group, similarly loaded with NAC, was also lower but did not reach significance after Bonferroni correction (n=8) for multiple comparisons.

Summary of plasma valine results in challenge study

- Plasma valine levels in this study were similar to those previously reported.
- A fall in plasma valine levels was generally observed by 270 minutes after oral loading with NAC or methionine irrespective of the group studied.
- Fasting mean plasma valine concentration in CP is lower than in patients with RAP. These differences persisted for the 9 hour profile and are reflected in differences in AUC in the methionine challenge pre-AOT.
- 10 weeks AOT appeared to have no effect on plasma valine profiles.

	Phase		Control	RAP	CP	Allpanc		t tests		
	Before AOT	**means**	246.1	252.2	186.5	225.9	c v cp	0.026	c v allp1	0.400
Both genders		**sd**	51.5	38.4	28.5	47.4	c v rap	0.797		
	After AOT	**means**		244.7	195.3	223.5	rap1 v cp1	0.002		
		sd		52.6	21.9	48.1	rap2 v cp2	0.038	c v allp2	0.354
	Before AOT	**means**	272.1	243.1	195.1	219.1	c v cp	0.0004	c v allp1	0.004
Male		**sd**	14.5	44.7	21.5	41.6	c v rap	0.230		
	After AOT	**means**		247.0	208.0	229.6	rap1 v cp1	0.076		
		sd		64.8	10.7	50.6	rap2 v cp2	0.253	c v allp2	0.043
	Before AOT	**means**	211.3	263.6	143.5	239.6	c v cp	N/A	c v allp1	0.580
Female		**sd**	66.8	31.0	N/A	60.1	c v rap	0.308		
	After AOT	**means**		240.8	170.0	212.5	rap1 v cp1	N/A		
		sd		35.3	11.3	60.1	rap2 v cp2	0.060	c v allp2	0.980

t tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.253	0.444	N/A
After AOT		0.869	0.059

Plasma valine (μmol/l)	**Before AOT**		**After AOT**	
	mean	**sd**	**mean**	**sd**
control nac	234.4	47.2		
control meth	250.9	59.4		
rap nac	241.0	33.1	252.1	57.5
rap meth	263.4	54.0	237.2	51.4
cp nac	183.6	40.7	192.7	33.2
cp meth	183.7	32.9	198.0	33.1

Table 4.8.3.4.1. **Mean baseline plasma Valine (μmol/l) in single- and mixed-gender groups.**

	Time (mins)	0	30	60	90	180	270	360	540	AUC
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control	mean	250.9	233.6	233.0	223.3	200.8	185.8	213.1	220.0	1925
	sd	59.4	59.2	68.5	58.8	58.9	51.9	59.0	46.9	516
RAP	mean	263.4	253.0	246.8	229.6	211.8	215.1	222.0	261.0	2065
	sd	54.0	49.1	45.2	41.0	44.4	35.3	38.2	41.5	300
CP	mean	183.7	172.3	176.4	173.1	146.9	136.0	176.0	192.1	1515
	sd	32.9	27.8	26.2	16.1	21.6	24.3	25.9	44.5	184
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP	mean	237.2	242.3	229.0	223.5	192.0	184.7	207.2	226.0	1889
	sd	51.4	52.4	42.6	33.7	40.6	40.5	27.3	32.4	290
CP	mean	198.0	195.2	183.0	183.5	167.5	162.3	192.2	200.5	1650
	sd	33.1	24.4	48.4	45.0	48.4	47.0	45.9	38.0	366
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control	mean	234.4	240.0	209.4	195.8	191.6	205.9	224.6	235.4	1916
	sd	47.2	57.4	41.5	41.7	39.9	43.9	53.9	51.8	355
RAP	mean	241.0	231.6	221.4	205.3	183.0	201.4	226.4	239.8	1938
	sd	33.1	25.1	28.1	26.3	22.4	37.2	48.0	43.0	235
CP	mean	183.6	194.4	179.1	174.4	155.6	155.0	177.3	180.7	1543
	sd	40.7	39.4	38.1	34.4	36.3	39.6	28.2	30.2	257
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP	mean	252.1	244.0	224.1	215.6	184.3	187.3	234.3	241.8	1977
	sd	57.5	48.5	42.5	41.5	35.2	37.0	51.0	41.5	331
CP	mean	192.7	174.3	179.3	156.3	160.0	156.5	169.0	170.7	1492
	sd	33.2	30.1	32.5	31.9	31.3	40.8	41.4	45.8	320

Table 4.8.3.4.2. **Group mean (s.d.) plasma valine (μmol/l) and AUC (μmol/l/hr) in challenge studies**

		t-tests - comparison of mean plasma valine concentration							
Challenge	**Groups**	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**
meth1 v meth1	c v rap	0.669	0.478	0.638	0.805	0.370	0.210	0.723	0.078
meth1 v meth1	c v cp	0.029	0.025	0.058	0.049	0.091	0.035	0.139	0.260
meth1 v meth2	c v rap	0.644	0.761	0.890	0.992	0.992	0.963	0.801	0.771
meth1 v meth2	c v cp	0.072	0.129	0.136	0.178	0.420	0.396	0.469	0.407
meth1 vs meth1	rap v cp	0.004	**0.001**	**0.002**	0.005	**0.001**	**0.0002**	0.013	0.008
meth2 v meth 2	rap v cp	0.109	0.048	0.094	0.101	0.340	0.373	0.497	0.216
meth1 v meth2	rap v rap	0.321	0.672	0.416	0.749	0.243	0.131	0.369	0.070
meth1 v meth2	cp v cp	0.469	0.142	0.774	0.612	0.368	0.254	0.467	0.722
NAC1 vs NAC1	c v rap	0.746	0.714	0.501	0.587	0.600	0.827	0.944	0.905
NAC1 vs NAC1	c v cp	0.043	0.095	0.165	0.298	0.090	0.035	0.070	0.021
NAC1 v NAC2	c v rap	0.511	0.883	0.494	0.374	0.701	0.409	0.728	0.969
NAC1 v NAC2	c v cp	0.076	0.018	0.155	0.068	0.123	0.052	0.060	0.022
NAC1 vs NAC1	rap v cp	0.011	0.058	0.032	0.074	0.111	0.033	0.024	0.006
NAC2 v NAC 2	rap v cp	0.033	0.007	0.045	0.014	0.200	0.200	0.022	0.013
NAC1 v NAC2	rap v rap	0.640	0.535	0.882	0.582	0.933	0.485	0.751	0.925
NAC1 v NAC2	cp v cp	0.666	0.321	0.992	0.347	0.818	0.948	0.689	0.658

meth 1 - methionine load pre-AOT
meth 2 - methionine load post-AOT

NAC 1 -NAC load pre-AOT
NAC 2 - NAC load post-AOT

c - Controls
rap - Recurrent acute pancreatitis
cp - Chronic pancreatitis

Table 4.8.3.4.3. **Students t-test (paired as appropriate) of group mean plasma valine (μmol/l).**

Figure 4.8.3.4.1. **Mean (+/- 1 s.d.) plasma valine profiles: control group data**

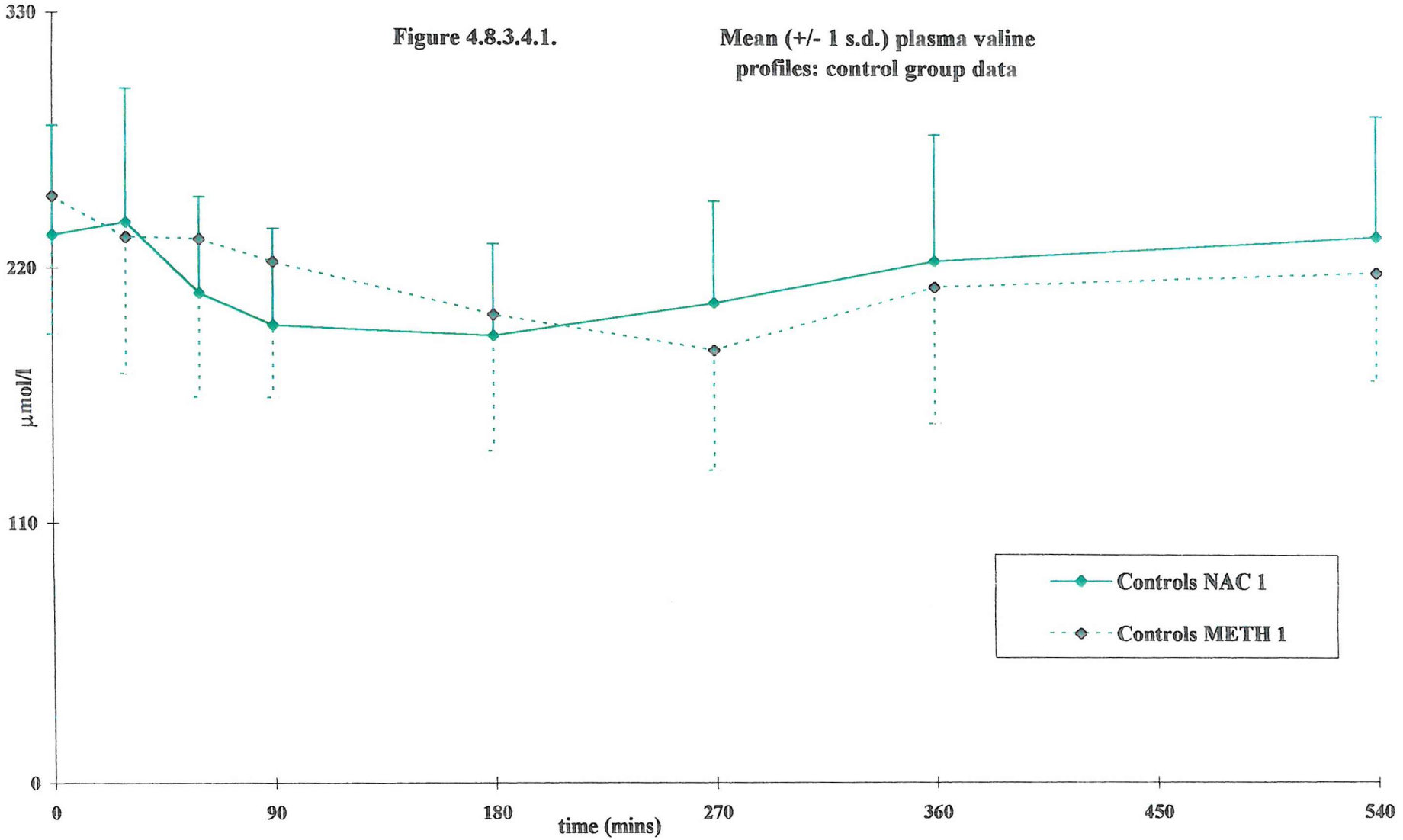


Figure 4.8.3.4.2. **Mean (+/- 1 s.d.) plasma valine profiles: RAP group data**

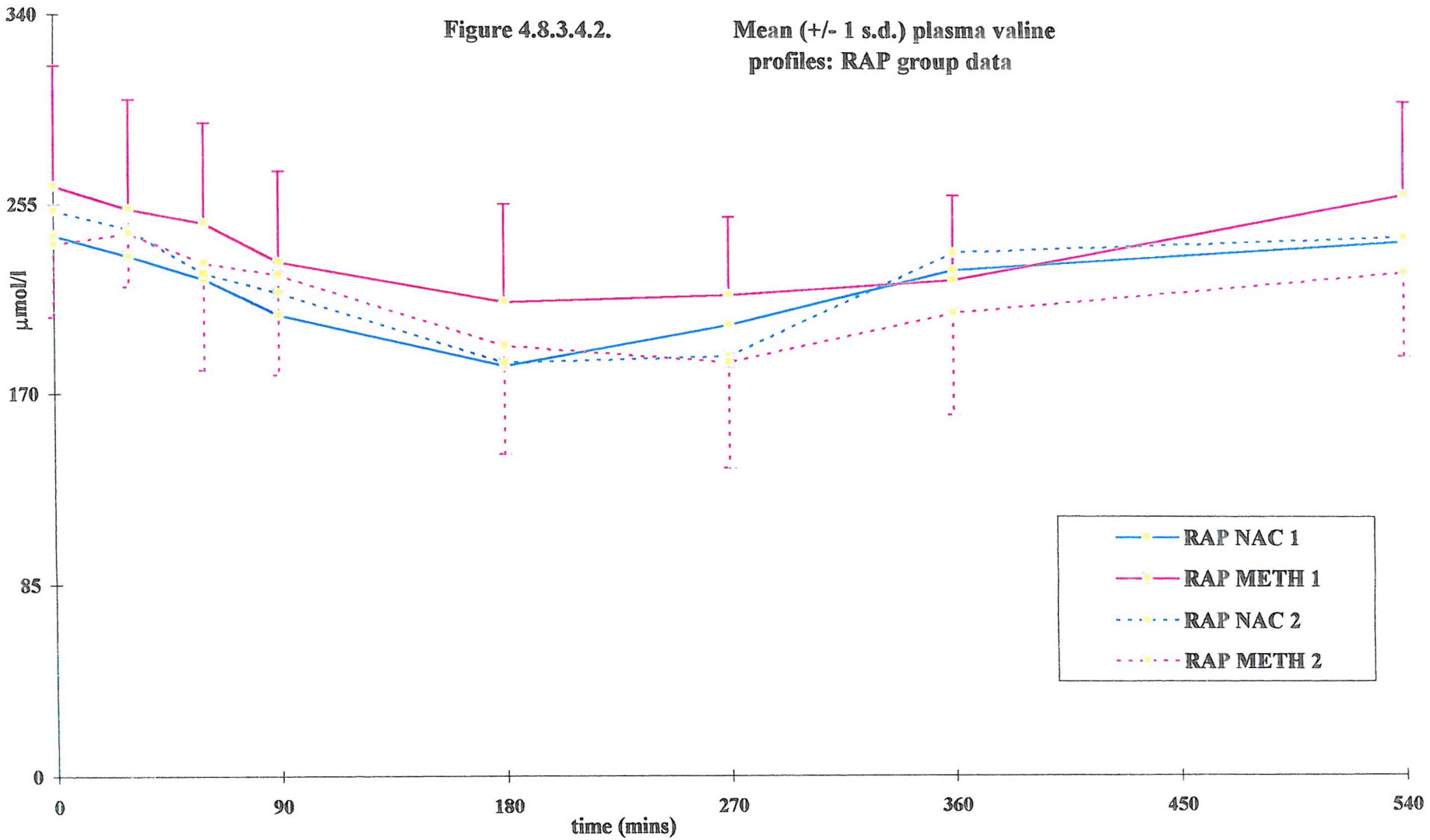


Figure 4.8.3.4.3. **Mean plasma valine profiles: CP group data (+/- 1 sd)**

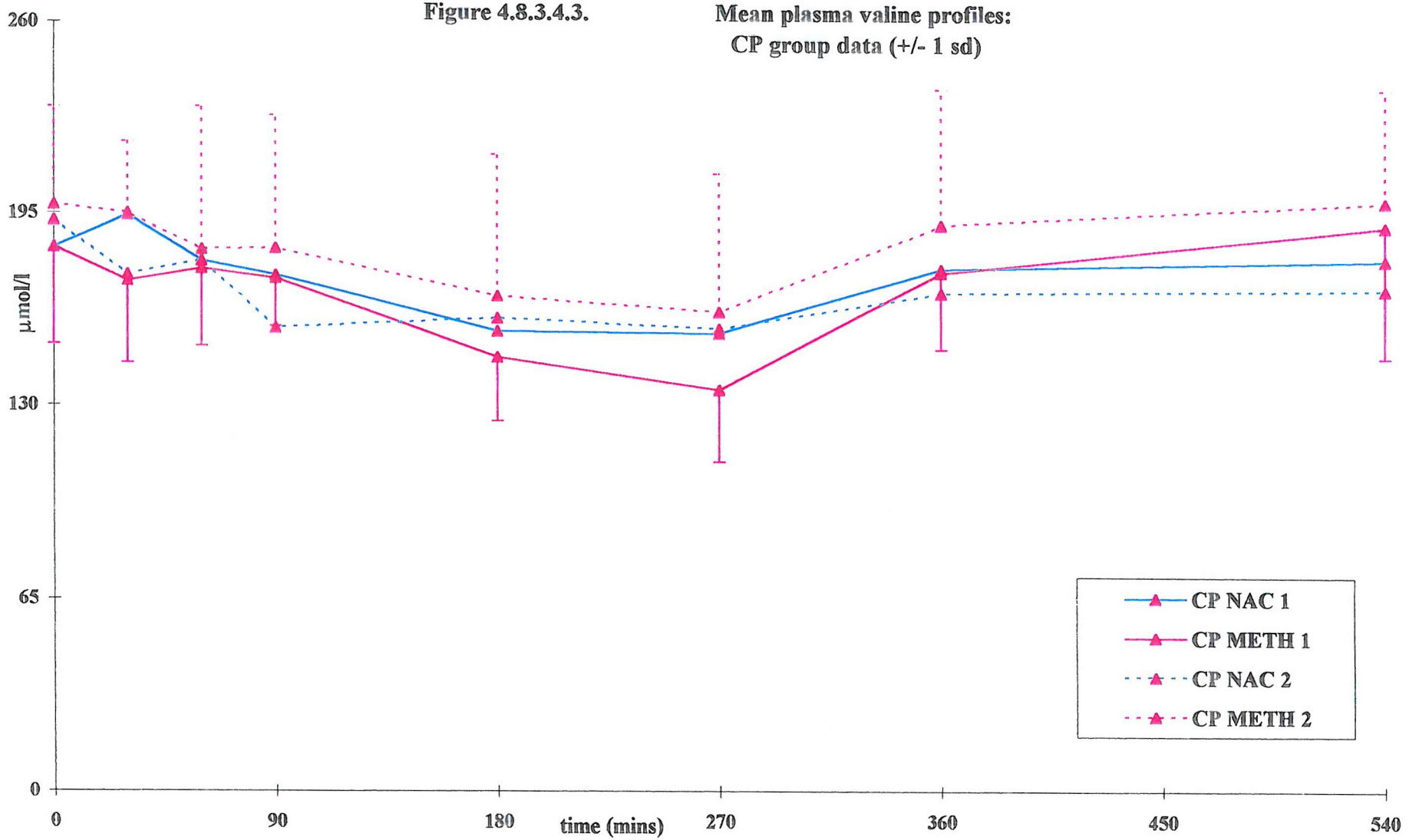


Figure 4.8.3.4.4. **Mean plasma valine profiles after oral NAC loading (+/- 1sd)**

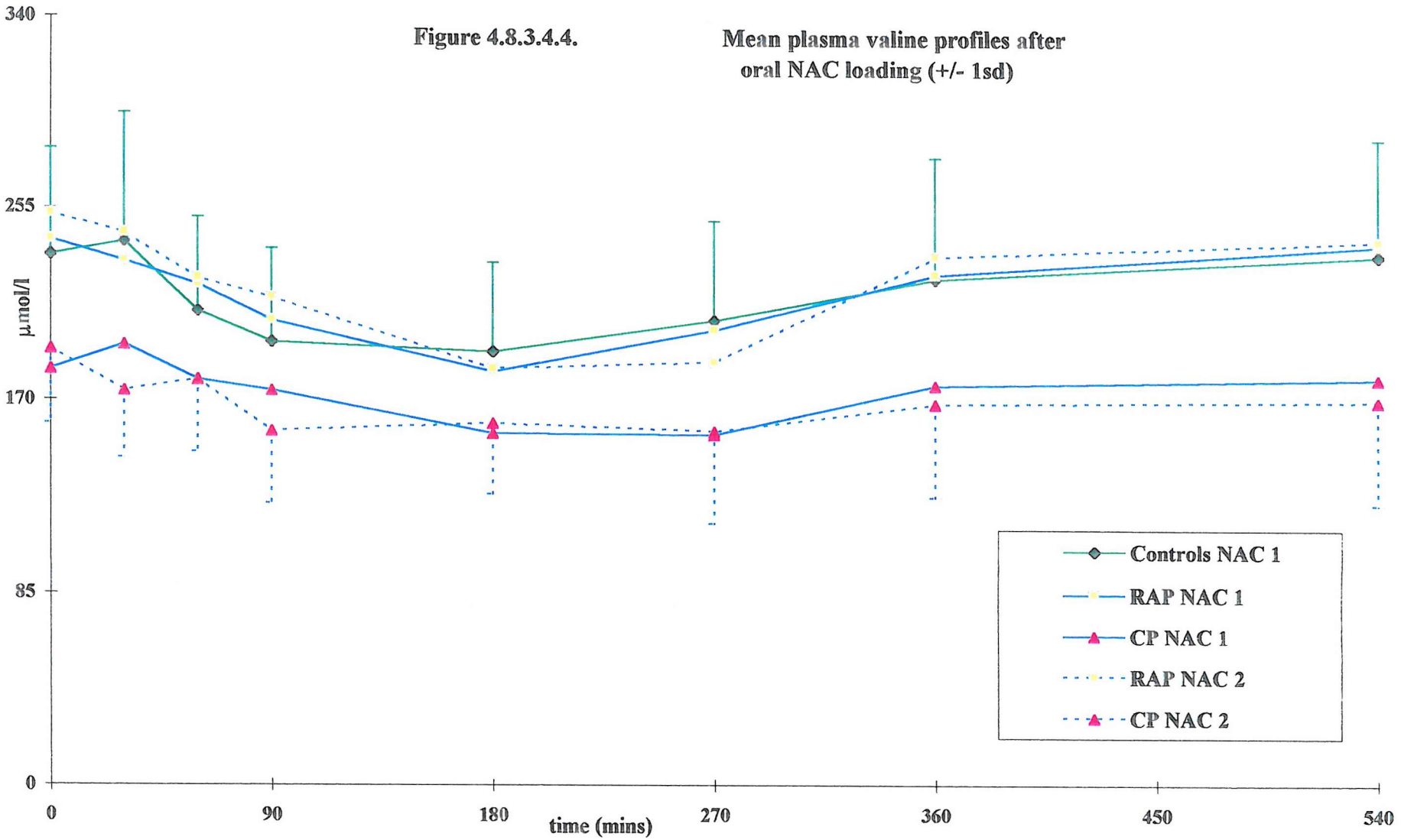
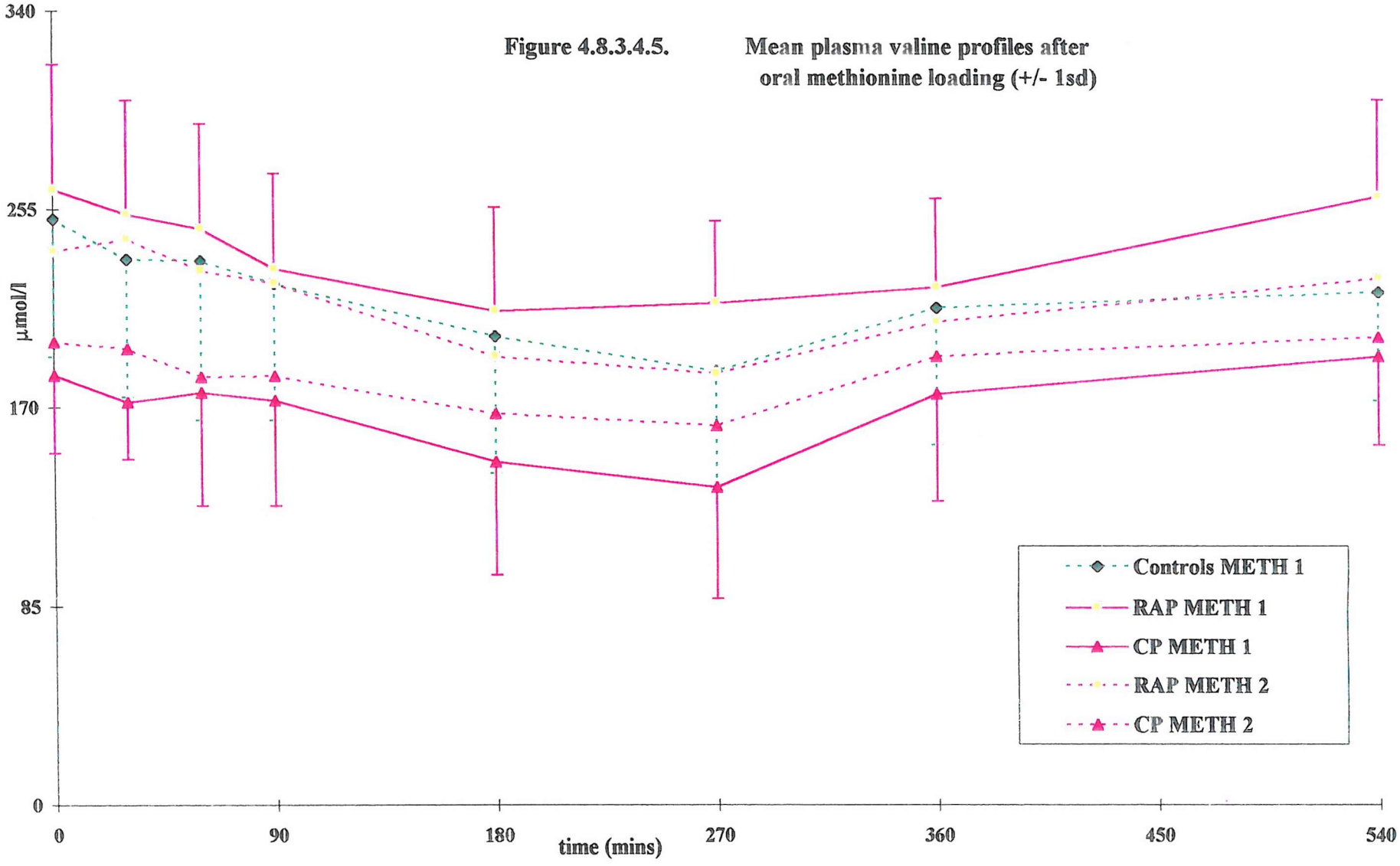


Figure 4.8.3.4.5. Mean plasma valine profiles after oral methionine loading (+/- 1sd)



	Time (x)						
	30	**60**	**90**	**180**	**270**	**360**	**540**
cn0 v cn(x)	0.4746	0.0239	**0.0026**	0.0058	0.0718	0.4764	0.6662
cm0 v cm(x)	0.2696	0.1240	0.0147	**0.0022**	**0.0005**	0.0332	0.0965
rn0 v rn(x)	0.0528	0.0904	0.0061	**0.0002**	0.0537	0.4787	0.8583
rm0 v rm(x)	0.0700	0.0075	0.0060	**0.0017**	0.0197	0.0445	0.9099
r2n0 v r2n(x)	0.0701	**0.0037**	0.0122	**0.0021**	0.0071	0.3312	0.4353
r2m0 v r2m(x)	0.3834	0.2554	0.2252	0.0064	**0.0013**	0.0333	0.5146
cpn0 v cpn(x)	0.2153	0.5349	0.2582	0.0642	0.1260	0.7398	0.7816
cpm0 v cpm(x)	0.1684	0.4499	0.5316	0.0334	0.0194	0.3281	0.5680
cp2n0 v cp2n(x)	0.1081	0.0272	**0.0029**	**0.0049**	0.0068	0.0384	0.0875
cp2m0 v cp2m(x)	0.7067	0.2680	0.0768	0.0678	0.0348	0.7252	0.8476

c - control
r - RAP pre-AOT
r2 - RAP post-AOT
cp - CP pre-AOT
cp2 - CP post-AOT

n - NAC load
m - methionine load
0 - time zero
(x) - time (x)

Table 4.8.4.3.4. **Paired-t-test of plasma valine at each profile time-pont versus baseline value**

	NAC1	NAC2	METH1	METH2
c	1843		2248	
c	1421		1231	
c	1507		1225	
c	2190		2223	
c	1835		1865	
c	1967			
c	2052		2140	
c	2518		2543	
mean	*1933.6*		*1908.6*	
rap	2127	2340	2115	2236
rap	2113	1611	1984	1925
rap	1865	1934	2128	1908
rap	1699	1747	1485	1501
rap	1663	2302	2504	1968
rap	2327	2122	2289	2080
rap	2010	1493	2143	1406
rap	1984	2271	2201	2086
rap	1659		1742	
mean	*1938.4*	*1977.2*	*2065.5*	*1888.6*
cp	1480	1189	1523	2364
cp	1500	1553	1270	1665
cp	1934		1473	
cp	1407	1312		1405
cp	1173	1169	1482	1381
cp	1823	1854	1503	1585
cp	1484	1877	1840	1499
mean	*1542.9*	*1492.3*	*1515.1*	*1650.0*

		t-tests			t-tests
meth1 vs meth1	rap v cp	0.001	NAC1 vs NAC1	rap v cp	0.008
meth2 v meth 2	rap v cp	0.219	NAC2 v NAC 2	rap v cp	0.018
meth1 v meth2	rap v rap	0.070	NAC1 v NAC2	rap v rap	0.979
meth1 v meth2	cp v cp	0.441	NAC1 v NAC2	cp v cp	0.879
meth1 v meth1	c v rap	0.537	NAC1 vs NAC1	c v rap	0.992
meth1 v meth1	c v cp	0.087	NAC1 vs NAC1	c v cp	0.036
meth1 v meth2	c v rap	0.873	NAC1 v NAC2	c v rap	0.729
meth1 v meth2	c v cp	0.288	NAC1 v NAC2	c v cp	0.036

Table 4.8.3.4.5. **Individual and mean AUC (μmol/l/hr) for valine in challenge studies**

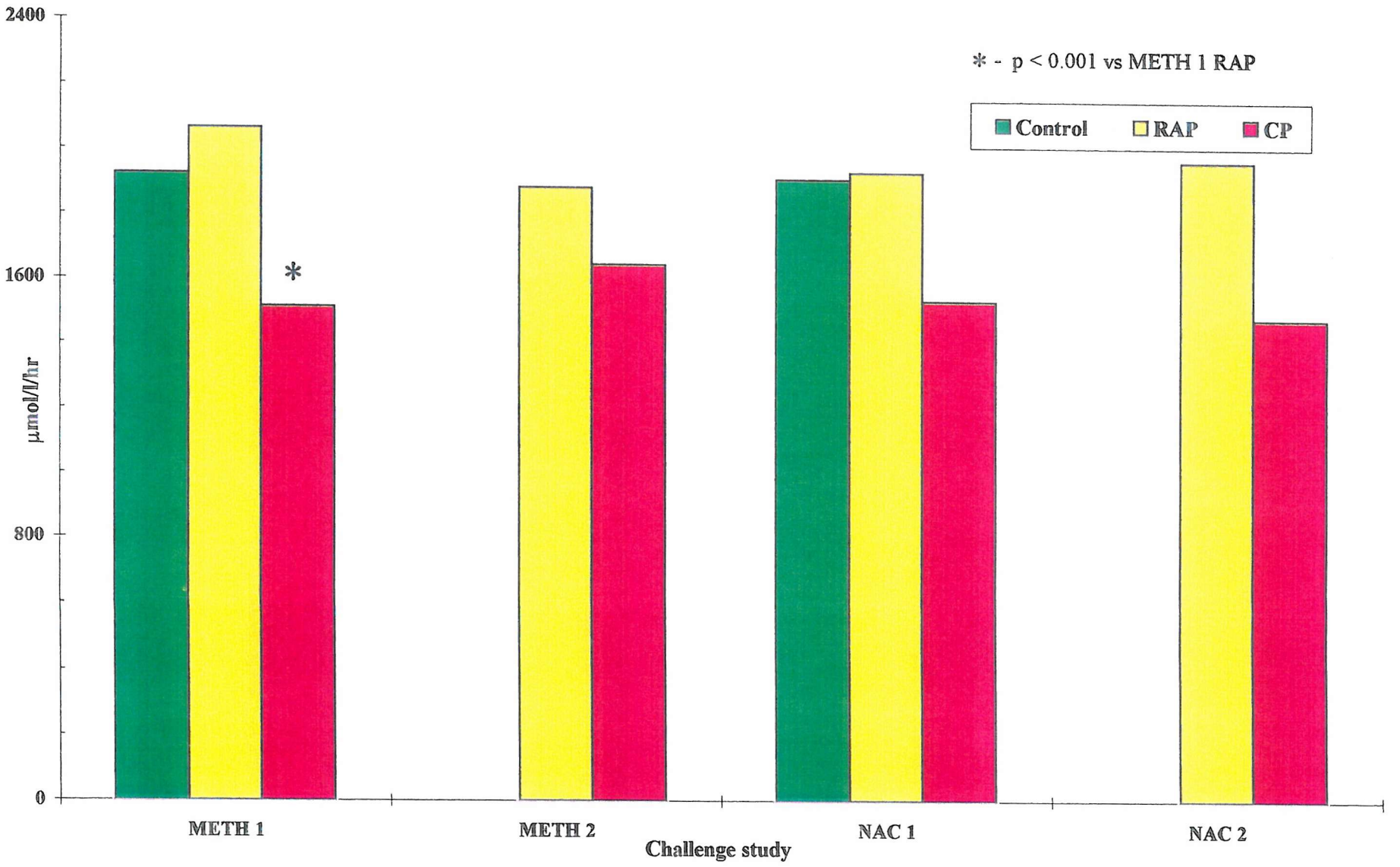


Table 4.8.3.4.6. Group mean AUC (µmol/l/hr) for plasma valine in challenge studies

4.8.3.5. Plasma Glycine

Mean fasting baseline plasma glycine concentrations were found to be within the reported normal range (Table 4.8.3.12.1). Control subject EGrY, a 34 year old woman, had a fasting plasma glycine of 510 μmol/l on the day she was loaded with NAC (Appendix C). This compared with a mean value of 245 μmol/l for the remainder of the control group. Her subsequent profile remained around this value, which is above the reported reference range. A normal distribution could thus not be assumed and the calculations related to this challenge study have used non-parametric methods.

Females have been reported to have higher plasma glycine concentrations than males (Armstrong 1973). In common with this we found a combined female control group baseline concentration of 347.8 +/- 83.8 μmol/l (mean +/- 1 s.d.) and 206.4 +/- 27.3 μmol/l in males. However, this was not significant and no differences were found at baseline between any of the study groups when analysed in mixed or single gender groups, or before or after AOT, Table 4.8.3.5.1.

Figures 4.8.3.5.1. - 3. plot the profiles of mean plasma glycine in the three subject groups in each phase of the study. Figures 4.8.3.5.4. & 5. collate the profiles of the groups separated by challenge compound. Oral loading with NAC did not result in any demonstrable change in plasma glycine concentration during the subsequent 9 hour observation period, Table 4.8.3.5.2. However in the group with RAP, glycine levels fell following oral loading with methionine. This fall was less marked after ten weeks AOT, Figure 4.8.3.5.5. and Table 4.8.3.5.3. No differences in the AUC between any of the subject groups was noted either before or after AOT, Table 4.8.3.5.2..

Summary of plasma glycine results in challenge study

- Plasma glycine levels were similar in female and male subjects.
- Oral NAC has no effect on plasma glycine profiles.

- Following oral methionine plasma glycine concentrations fell in patients with RAP.
- No difference in the AUC of the plasma glycine profiles in the challenge studies, was seen between group irrespective of AOT status.

Phase		Control	RAP	CP	Allpanc	Wilcoxon / t-tests			
Before AOT	**means**	267.0	248.1	238.9	244.2	c v cp	0.516	c v allp1	0.569
Both genders	**sd**	87.0	91.1	63.4	77.7	c v rap	0.688		
After AOT	**means**		245.6	224.1	236.4	rap1 v cp1	0.827		
	sd		71.8	65.8	67.6	rap2 v cp2	0.572	c v allp2	0.434
Before AOT	**means**	210.1	248.3	244.2	246.0	c v cp	0.349	c v allp1	0.198
Male	**sd**	25.1	83.5	69.4	70.9	c v rap	0.435		
After AOT	**means**		233.7	220.0	227.6	rap1 v cp1	0.941		
	sd		67.1	84.6	70.6	rap2 v cp2	0.801	c v allp2	0.516
Before AOT	**means**	347.8	248.0	212.5	240.9	c v cp	N/A	c v allp1	0.118
Female	**sd**	83.8	111.3	N/A	97.7	c v rap	0.201		
After AOT	**means**		265.5	232.3	252.2	rap1 v cp1	N/A		
	sd		90.0	0.4	97.7	rap2 v cp2	0.588	c v allp2	0.115

Wilcoxon / t-tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.0598	0.997	N/A
After AOT		0.629	0.341

Table 4.8.3.5.1. **Time Zero Plasma Glycine (μmol/l) in single and mixed-gender groups**

Figure 4.8.3.5.1. **Mean plasma glycine profiles: control group data (+/- 1sd)**

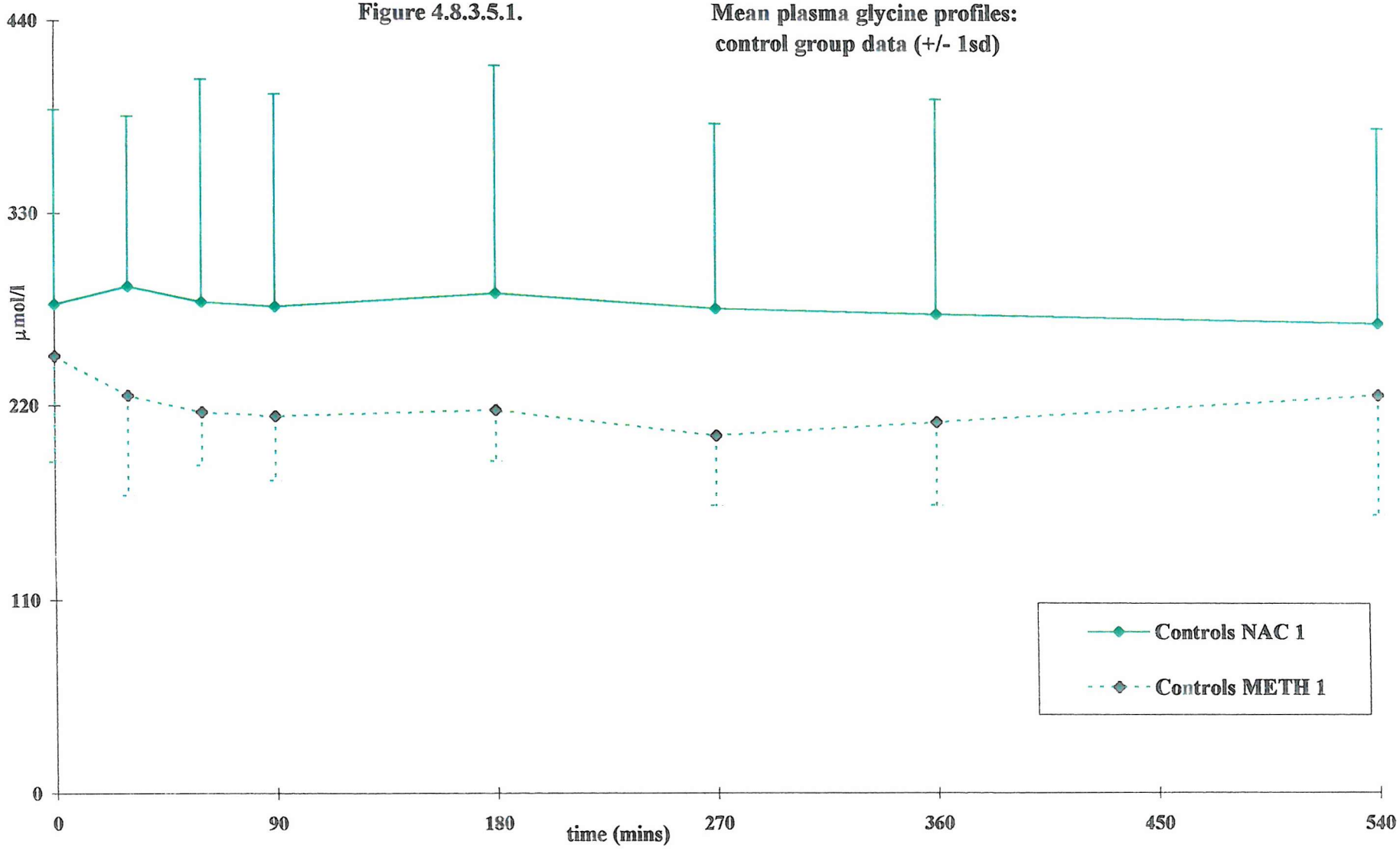


Figure 4.8.3.5.2. **Mean plasma glycine profiles: RAP group data (+/- 1 s.d.)**

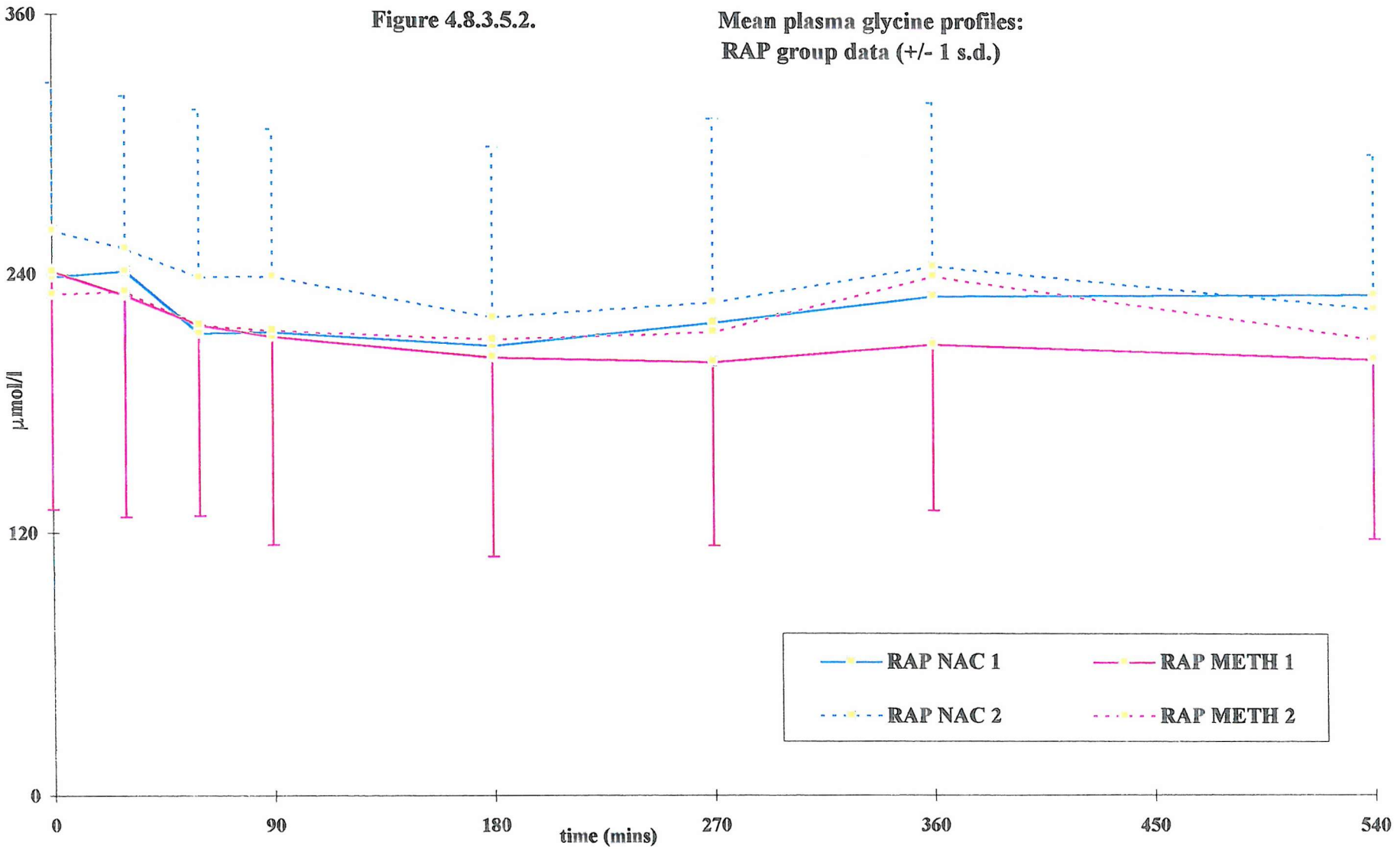


Figure 4.8.3.5.3. **Mean plasma glycine profiles: CP group data (+/- 1 s.d.)**

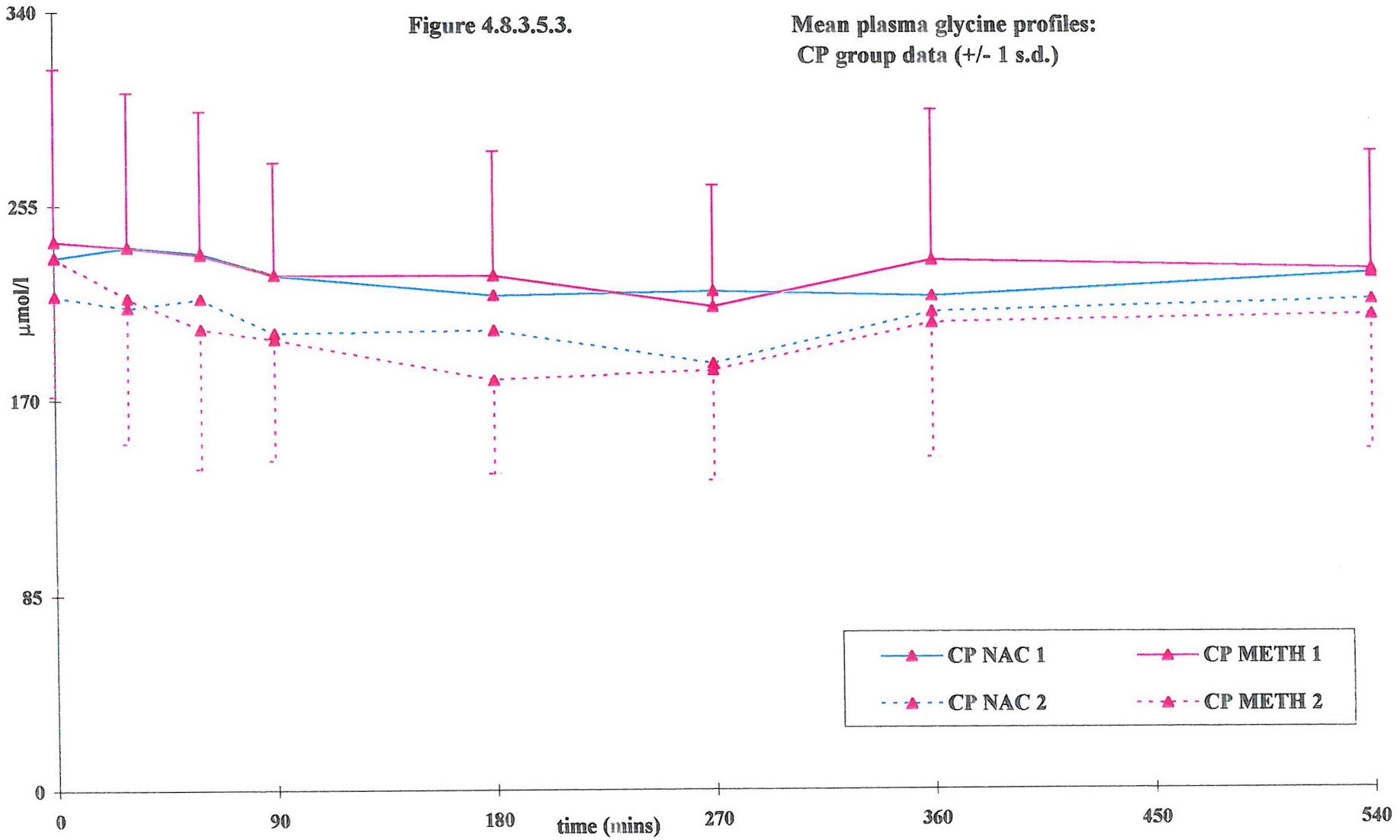


Figure 4.8.3.5.4. Mean plasma gycine profiles after oral NAC loading (+/- 1sd)

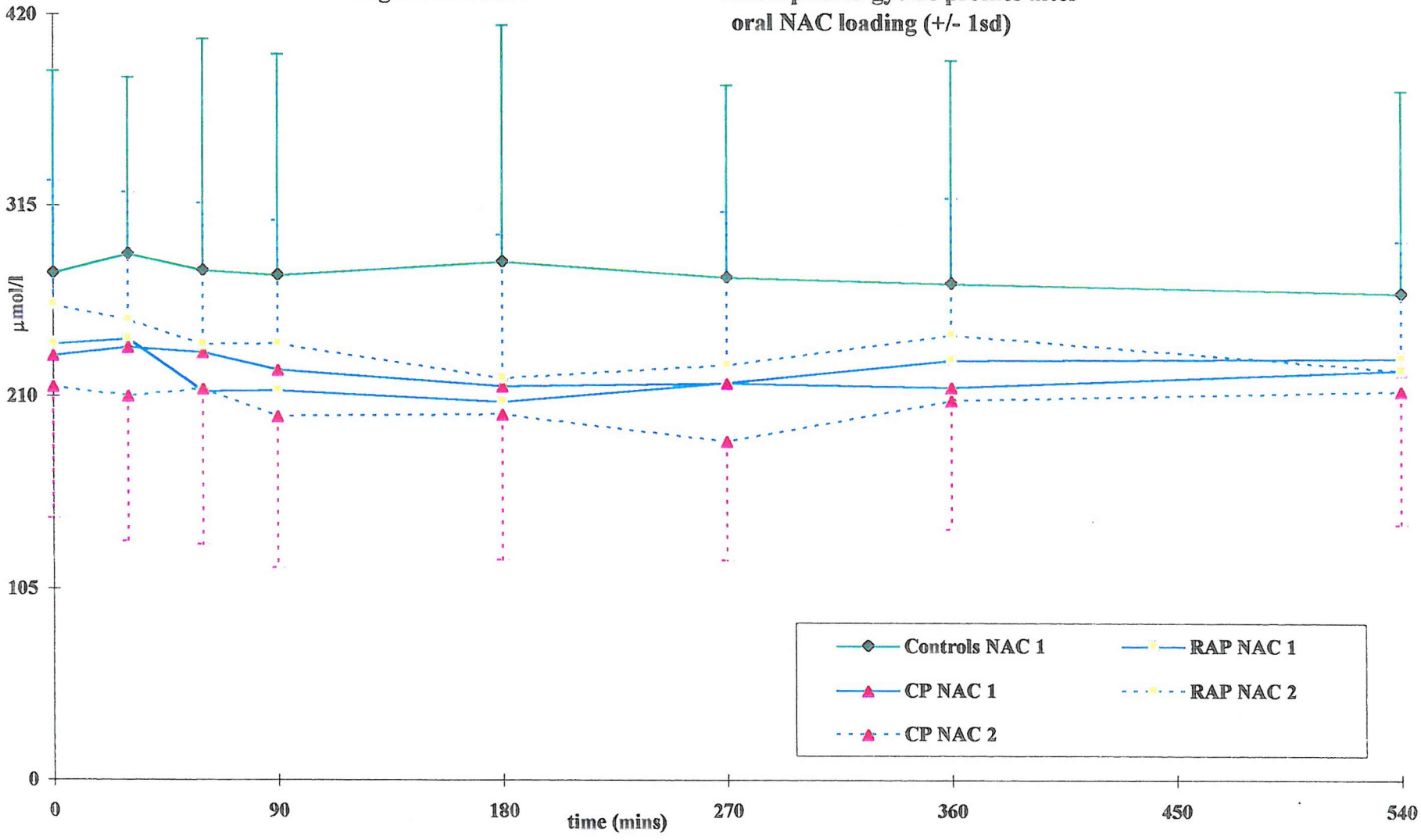


Figure 4.8.3.5.5. **Mean plasma glycine profiles after oral methionine loading (+/- 1sd)**

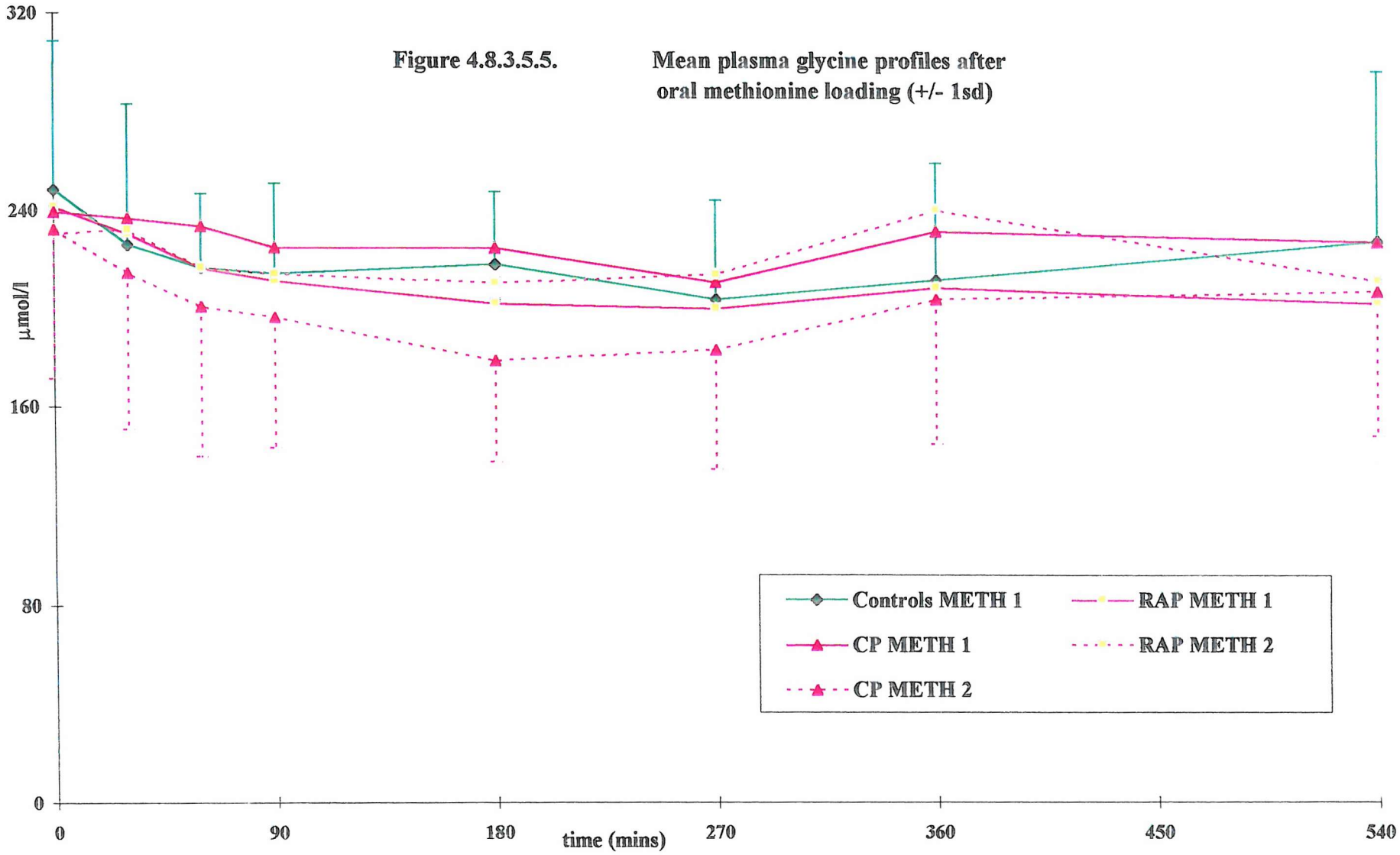


Table 4.8.3.5.2. **Group mean plasma glycine (μmol/l) and AUC (μmol/l/hr) in challenge studies**

	Time (mins)	0	30	60	90	180	270	360	540	AUC
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control	mean	248.3	226.0	216.6	214.5	218.4	204.1	212.1	228.1	1958
	sd	60.3	57.1	30.2	36.6	29.3	40.2	47.4	68.8	404
RAP	mean	241.6	230.7	216.9	211.8	202.5	200.6	209.1	203.0	1869
	sd	69.2	77.3	69.1	63.4	59.3	64.7	56.4	61.0	534
CP	mean	239.5	237.0	233.9	225.1	225.1	211.3	232.0	228.1	2047
	sd	75.9	68.0	62.9	49.2	54.5	53.3	65.8	51.5	538
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP	mean	230.7	232.3	217.0	214.5	210.8	214.5	240.6	212.3	1995
	sd	81.4	80.4	82.5	80.2	74.8	63.9	72.4	64.2	622
CP	mean	232.5	214.8	201.2	196.8	179.2	183.7	204.5	208.0	1779
	sd	61.0	63.8	61.2	53.2	41.1	48.5	59.3	59.2	469
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control	mean	278.1	288.8	279.9	277.4	285.3	276.8	273.9	269.0	2499
	sd	111.4	97.3	127.4	121.7	130.1	106.0	122.9	111.6	1012
RAP	mean	238.9	241.8	213.2	213.8	207.8	218.7	231.3	232.8	2004
	sd	110.2	102.7	88.0	96.4	92.0	85.1	76.9	83.3	756
CP	mean	232.7	237.3	234.4	225.1	216.4	218.3	216.3	226.4	1984
	sd	52.5	72.8	63.1	65.2	68.6	72.5	76.9	58.3	590
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP	mean	260.5	252.4	238.9	239.4	220.8	228.2	245.3	226.1	2101
	sd	68.3	70.4	78.0	68.4	79.4	85.1	75.5	71.6	651
CP	mean	215.7	210.5	214.5	199.7	201.0	186.7	209.2	214.8	1841
	sd	71.8	79.5	85.1	82.3	79.2	65.0	70.6	73.3	650

Comparison	Time (x)						
	30	**60**	**90**	**180**	**270**	**360**	**540**
cn0 v cn(x)	0.342	0.846	0.924	0.491	0.898	0.930	0.412
cm0 v cm(x)	0.013	0.123	0.027	0.073	0.018	0.111	0.350
rn0 v rn(x)	0.240	0.058	0.069	0.023	0.263	0.747	0.682
rm0 v rm(x)	0.155	**0.004**	**0.001**	**0.003**	**0.002**	0.008	**0.001**
r2n0 v r2n(x)	0.114	0.023	0.021	0.007	0.037	0.366	0.017
r2m0 v r2m(x)	0.681	**0.005**	0.016	0.030	0.255	0.540	0.261
cpn0 v cpn(x)	0.729	0.863	0.619	0.299	0.322	0.443	0.668
cpm0 v cpm(x)	0.045	0.122	0.307	0.272	0.099	0.908	0.732
cp2n0 v cp2n(x)	0.618	0.929	0.260	0.087	0.034	0.438	0.955
cp2m0 v cp2m(x)	0.013	0.081	0.028	0.009	0.009	0.072	0.076

c - control
r - RAP pre-AOT
r2 - RAP post-AOT
cp - CP pre-AOT
cp2 - CP post-AOT

n - NAC load
m - methionine load
0 - time zero
(x) - time (x)

Table 4.8.3.5.3. Paired-t-test of plasma glycine at each profile time-point versus baseline value

4.8.3.6. Plasma Serine

Fasting plasma serine concentrations were also found to be within the reported normal range in all subjects, Table 4.8.3.12.1. Gender sub-group analysis revealed no differences between the male and female subjects, Table 4.8.3.6.1.

Figures 4.8.3.6.1. - 3. plot the profiles of plasma serine in the three subject groups observed after oral loading with NAC and methionine. Figures 4.8.3.6.4. & 5. collate the profiles of the three subject groups before and after AOT. Loading with methionine did not result in any demonstrable change in plasma serine concentration during the subsequent 9 hour observation period, Tables 4.8.3.6.2. and 3. Oral loading with NAC was associated with a rise in plasma serine after 30 minutes in the control group, but a fall by 270 minutes in individuals with RAP after antioxidant supplementation, Table 4.8.3.6.4.

The mean AUC for serine concentration in the three groups, over the challenge studies are shown in Table 4.8.3.6.2. and are plotted in Figure 4.8.3.6.6. No difference between the AUC of any of the subject groups was noted either before or after AOT.

Summary of plasma serine results in challenge study

- Plasma serine concentration alters after oral NAC, but at different time points and in opposite directions in diverse subject groups. It is unlikely that these differences are true.
- Plasma serine does not alter after oral methionine and the AUC of plasma serine profiles was the same irrespective of subject group, challenge compound and AOT status.

Phase		Control	RAP	CP	Allpanc	t tests			
Before AOT	means	114.9	103.1	93.2	99.1	c v cp	0.089	c v allp1	0.095
Both genders	sd	16.9	25.2	23.1	24.1	c v rap	0.282		
After AOT	means		108.7	97.2	103.8	rap1 v cp1	0.448		
	sd		17.5	27.9	22.4	rap2 v cp2	0.399	c v allp2	0.221
Before AOT	means	95.0	104.5	100.1	102.3	c v cp	0.761	c v allp1	0.662
Male	sd	31.4	31.0	17.6	23.9	c v rap	0.643		
After AOT	means		117.3	98.5	107.9	rap1 v cp1	0.791		
	sd		20.5	27.4	24.6	rap2 v cp2	0.316	c v allp2	0.459
Before AOT	means	122.8	101.4	58.5	92.8	c v cp	N/A	c v allp1	0.185
Female	sd	26.1	20.2	N/A	25.9	c v rap	0.308		
After AOT	means		103.7	103.3	103.5	rap1 v cp1	N/A		
	sd		8.5	27.9	25.9	rap2 v cp2	0.987	c v allp2	0.332

t tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.234	0.861	N/A
After AOT		0.292	0.601

Table 4.8.3.6.1. Time Zero Plasma Serine (μmol/l) in mixed-gender groups and by gender

Figure 4.8.3.6.1. Mean plasma serine profiles: control group data (+/- 1sd)

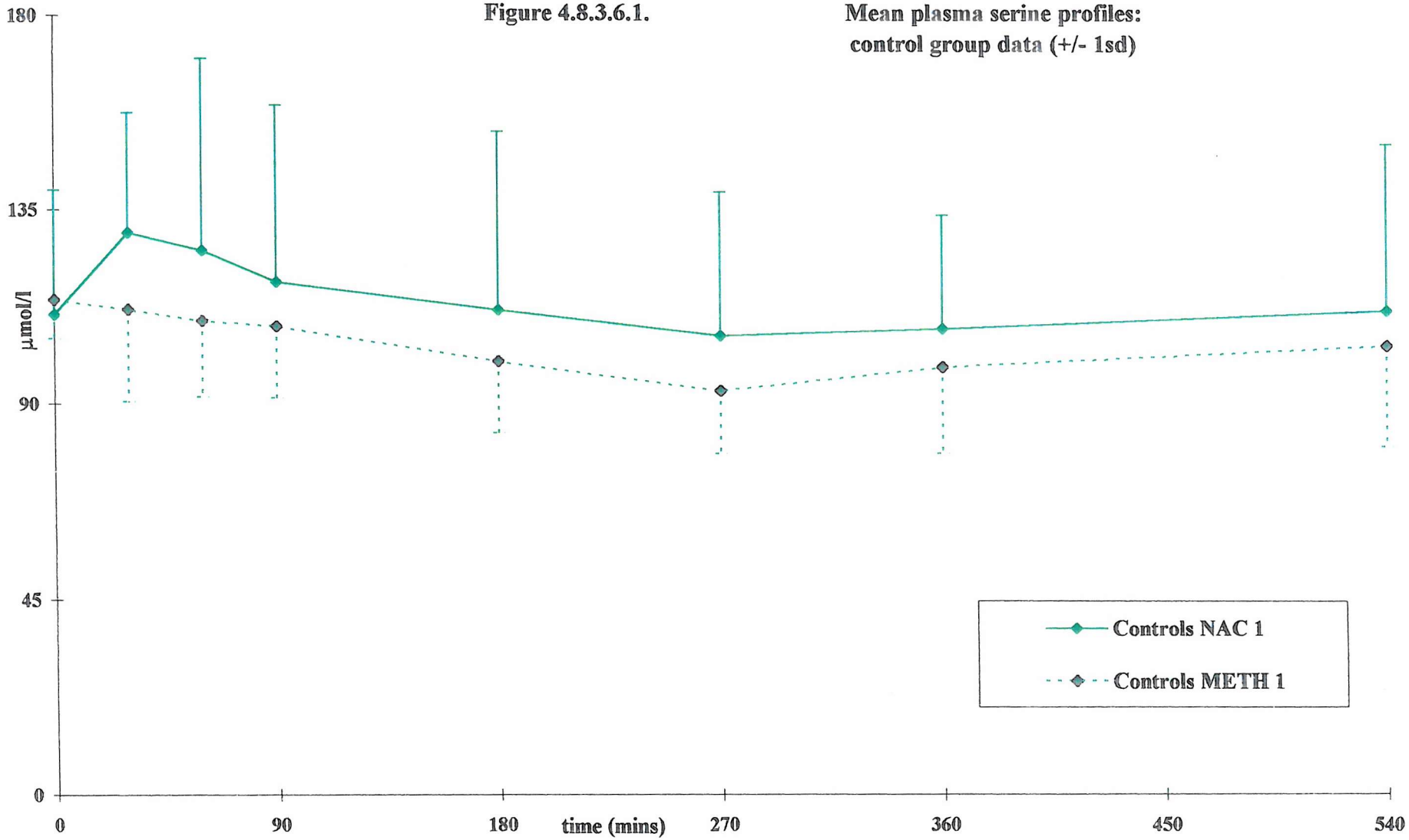


Figure 4.8.3.6.2.

Mean plasma serine profiles:
RAP group data (+/- 1 sd)

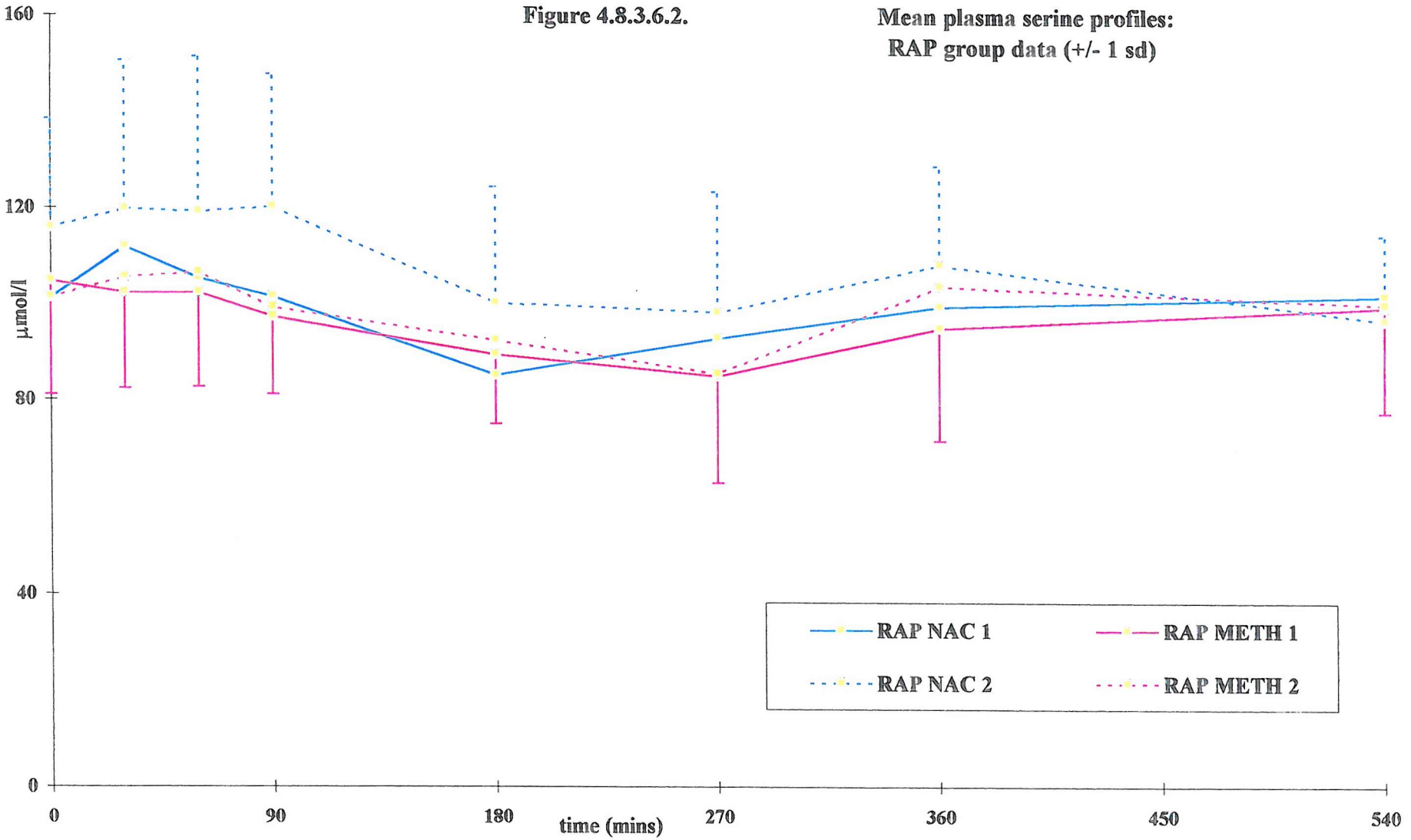


Figure 4.8.3.6.3.

Mean plasma serine profiles:
CP group data (+/- 1 s.d.)

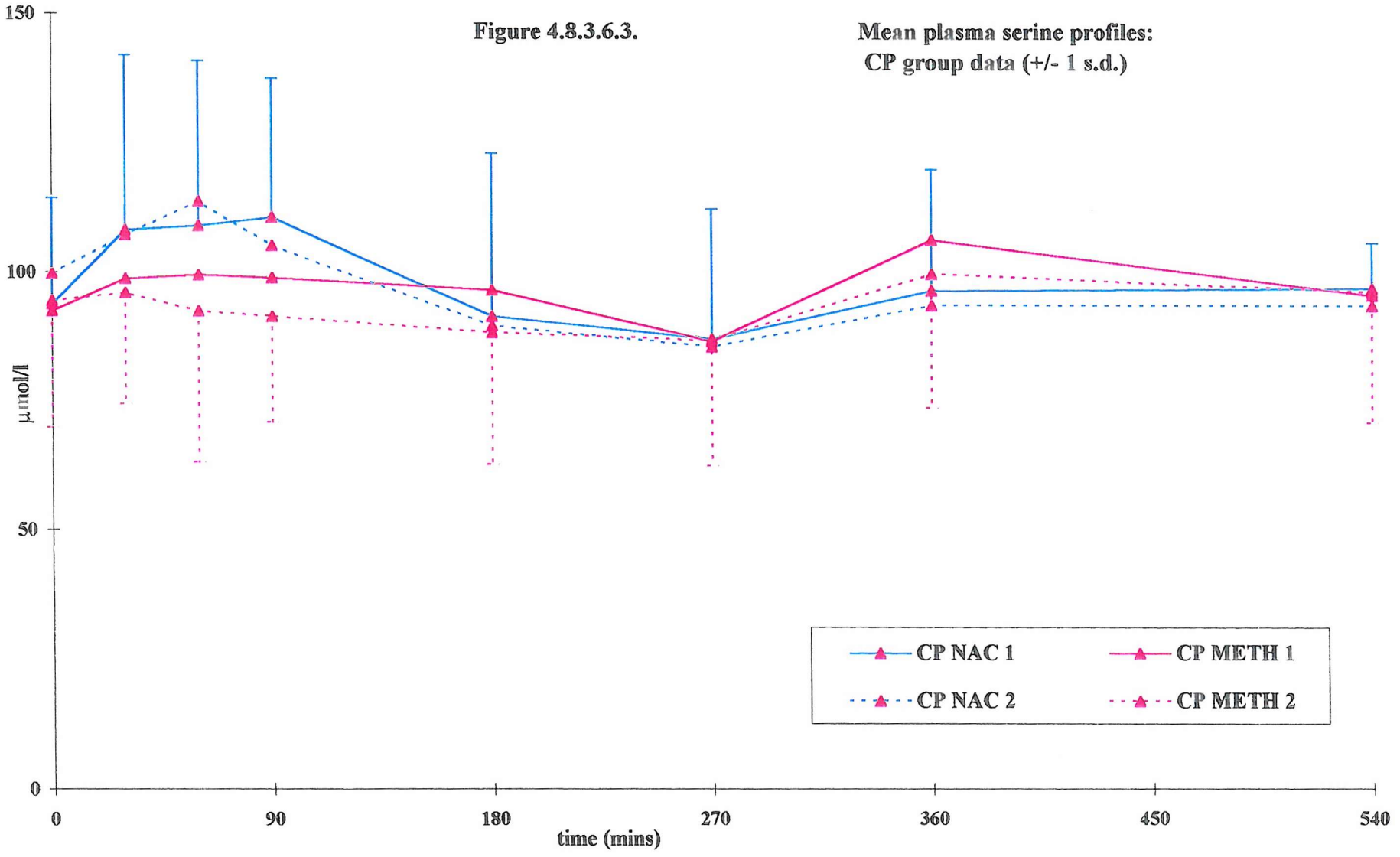


Figure 4.8.3.6.4. Mean plasma serine profiles: group data before AOT (+/- 1 s.d.)

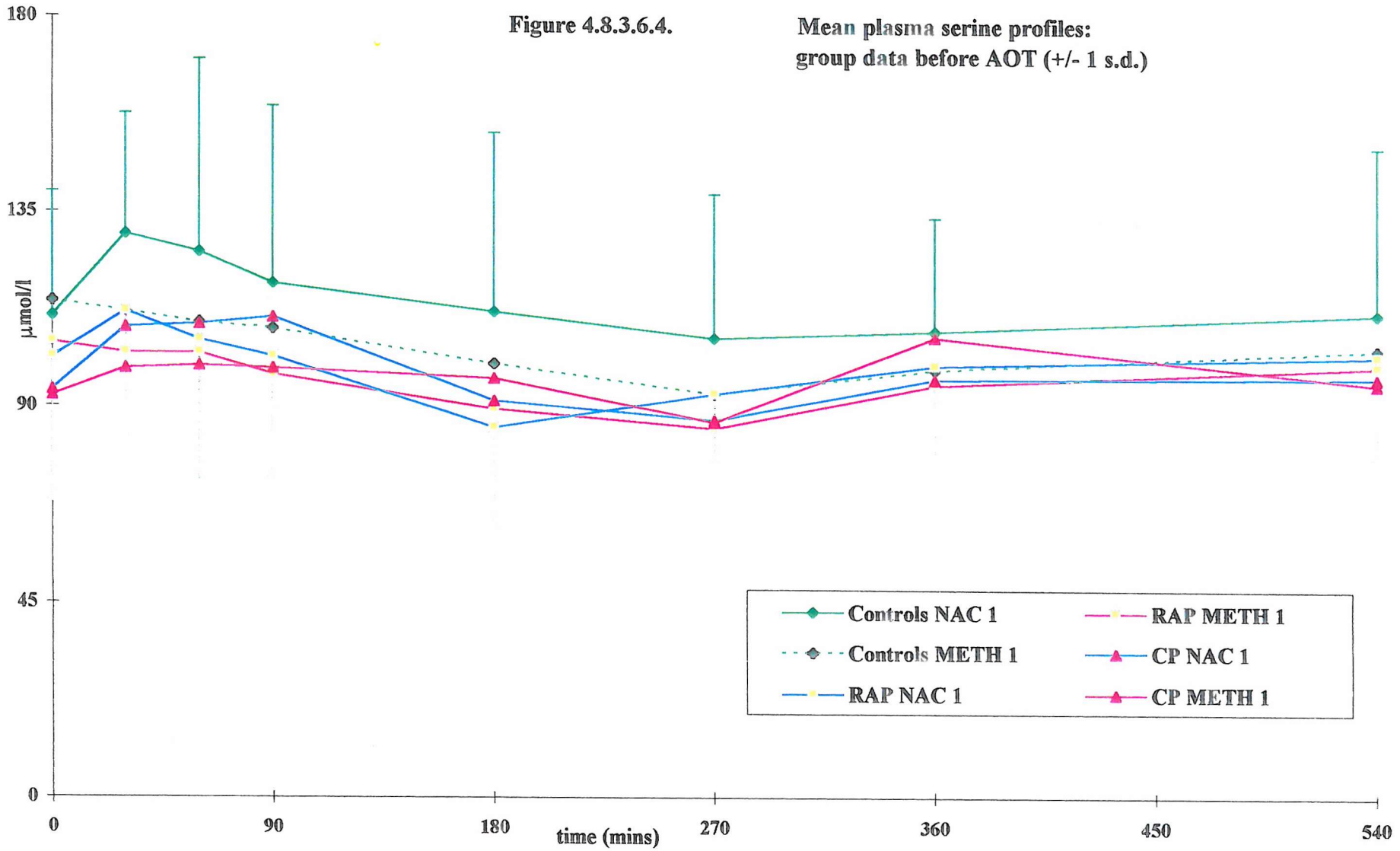
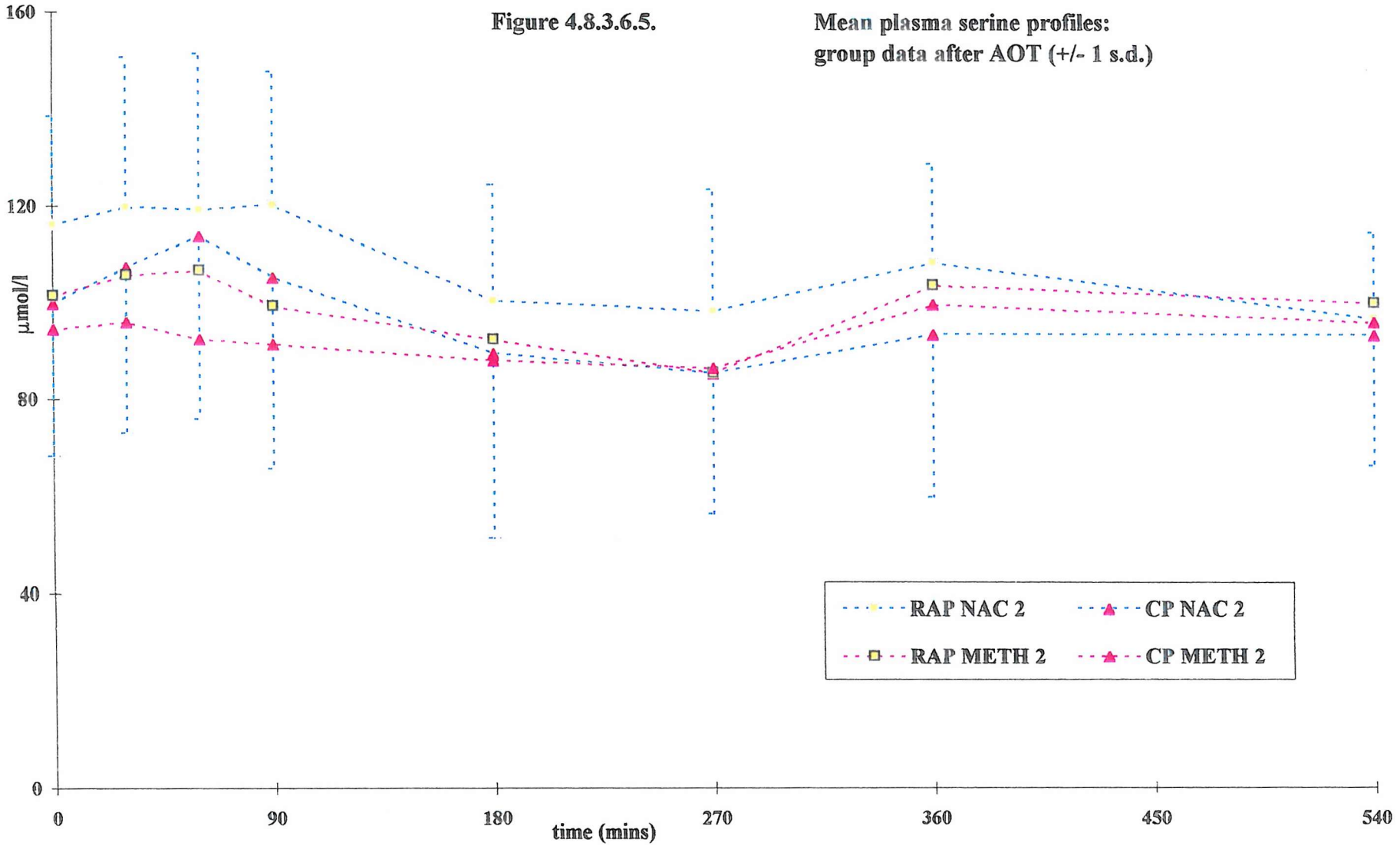


Figure 4.8.3.6.5. **Mean plasma serine profiles: group data after AOT (+/- 1 s.d.)**



	Time (mins)	0	30	60	90	180	270	360	540	AUC
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control	mean	114.3	112.0	109.4	108.1	100.1	93.3	98.9	103.9	950.0
	sd	9.1	21.5	17.7	16.7	16.8	14.6	20.1	23.4	113.4
RAP	mean	104.9	102.4	102.4	97.5	89.6	85.3	95.4	100.0	851.8
	sd	23.7	20.0	19.6	16.2	14.3	22.0	23.5	22.0	164.7
CP	mean	92.5	98.9	99.6	99.0	96.7	86.7	106.6	95.9	867.5
	sd	26.2	27.2	21.0	21.7	21.8	23.0	27.6	16.4	199.5
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP	mean	101.5	105.7	106.8	99.4	92.7	85.8	104.1	100.6	883.7
	sd	20.7	20.6	24.6	21.7	22.2	10.3	19.6	17.7	133.9
CP	mean	94.5	96.0	92.5	91.5	88.3	86.8	100.0	96.5	841.9
	sd	24.6	21.5	29.3	20.6	25.5	24.3	26.3	25.5	215.2
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control	mean	110.9	129.9	125.8	118.5	112.1	106.1	107.9	112.1	1012.7
	sd	28.8	28.0	44.4	41.0	41.4	33.4	26.3	38.6	285.9
RAP	mean	101.3	112.0	105.4	101.7	85.3	93.2	99.9	102.3	879.5
	sd	33.2	28.9	33.3	27.9	22.6	15.7	20.5	23.3	151.3
CP	mean	93.9	108.3	109.1	110.7	91.6	87.1	96.7	97.3	874.5
	sd	20.7	33.8	31.8	26.9	31.7	25.3	23.4	8.9	203.2
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP	mean	116.0	119.8	119.3	120.3	100.5	98.5	108.6	97.3	949.5
	sd	22.6	30.9	32.2	27.5	24.1	25.1	20.4	17.7	187.6
CP	mean	99.8	107.3	113.8	105.3	89.7	85.7	93.8	93.8	855.8
	sd	31.8	34.5	37.9	39.7	38.3	29.2	33.9	27.2	294.0

Table 4.8.3.6.2. **Group mean plasma serine (μmol/l) and AUC (μmol/l/hr) in challenge studies**

2 tailed t-test - comparison of mean plasma serine concentrations (p - values)									
		0	30	60	90	180	270	360	540
meth1 v meth1	c v rap	0.299	0.359	0.456	0.218	0.200	0.409	0.750	0.731
meth1 v meth1	c v cp	0.100	0.325	0.352	0.385	0.743	0.532	0.555	0.452
meth1 v meth2	c v rap	0.144	0.558	0.810	0.386	0.462	0.259	0.610	0.755
meth1 v meth2	c v cp	0.110	0.196	0.248	0.138	0.353	0.582	0.932	0.591
meth1 vs meth1	rap v cp	0.373	0.775	0.785	0.883	0.481	0.902	0.411	0.673
meth2 v meth 2	rap v cp	0.588	0.415	0.359	0.500	0.747	0.924	0.758	0.746
meth1 v meth2	rap v rap	0.755	0.747	0.698	0.843	0.752	0.952	0.423	0.954
meth1 v meth2	cp v cp	0.894	0.837	0.634	0.536	0.542	0.993	0.669	0.959
NAC1 vs NAC1	c v rap	0.536	0.229	0.311	0.348	0.133	0.342	0.522	0.545
NAC1 vs NAC1	c v cp	0.208	0.207	0.417	0.668	0.297	0.234	0.419	0.323
NAC1 v NAC2	c v rap	0.698	0.503	0.743	0.922	0.507	0.635	0.951	0.346
NAC1 v NAC2	c v cp	0.518	0.221	0.599	0.557	0.316	0.247	0.430	0.319
NAC1 vs NAC1	rap v cp	0.590	0.824	0.825	0.522	0.668	0.590	0.781	0.562
NAC2 v NAC 2	rap v cp	0.317	0.502	0.784	0.458	0.560	0.434	0.372	0.796
NAC1 v NAC2	rap v rap	0.301	0.613	0.399	0.205	0.203	0.660	0.394	0.618
NAC1 v NAC2	cp v cp	0.703	0.961	0.816	0.785	0.925	0.925	0.865	0.776

Table 4.8.3.6.3. **Students' t-tests - comparison of mean plasma serine concentration**

	Time (x)						
Comparison	**30**	**60**	**90**	**180**	**270**	**360**	**540**
cn0 v cn(x)	**0.001**	0.056	0.167	0.811	0.258	0.584	0.876
cm0 v cm(x)	0.552	0.941	0.678	0.048	0.003	0.064	0.443
rn0 v rn(x)	0.210	0.599	0.937	0.007	0.399	0.915	0.923
rm0 v rm(x)	0.546	0.542	0.022	0.033	0.066	0.093	0.553
r2n0 v r2n(x)	0.445	0.521	0.744	0.012	**0.002**	0.214	**0.001**
r2m0 v r2m(x)	0.027	0.174	0.586	0.054	0.009	0.765	0.911
cpn0 v cpn(x)	0.101	0.060	0.031	0.758	0.231	0.670	0.579
cpm0 v cpm(x)	0.027	0.422	0.354	0.570	0.143	0.250	0.850
cp2n0 v cp2n(x)	0.359	0.025	0.221	0.124	0.043	0.115	0.034
cp2m0 v cp2m(x)	0.713	0.662	0.542	0.410	0.278	0.262	0.504

c - control
r - RAP pre-AOT
r2 - RAP post-AOT
cp - CP pre-AOT
cp2 - CP post-AOT

n - NAC load
m - methionine load
0 - time zero
(x) - time (x)

Table 4.8.3.6.4. **Paired-t-test of plasma serine at each profile time-point versus baseline value**

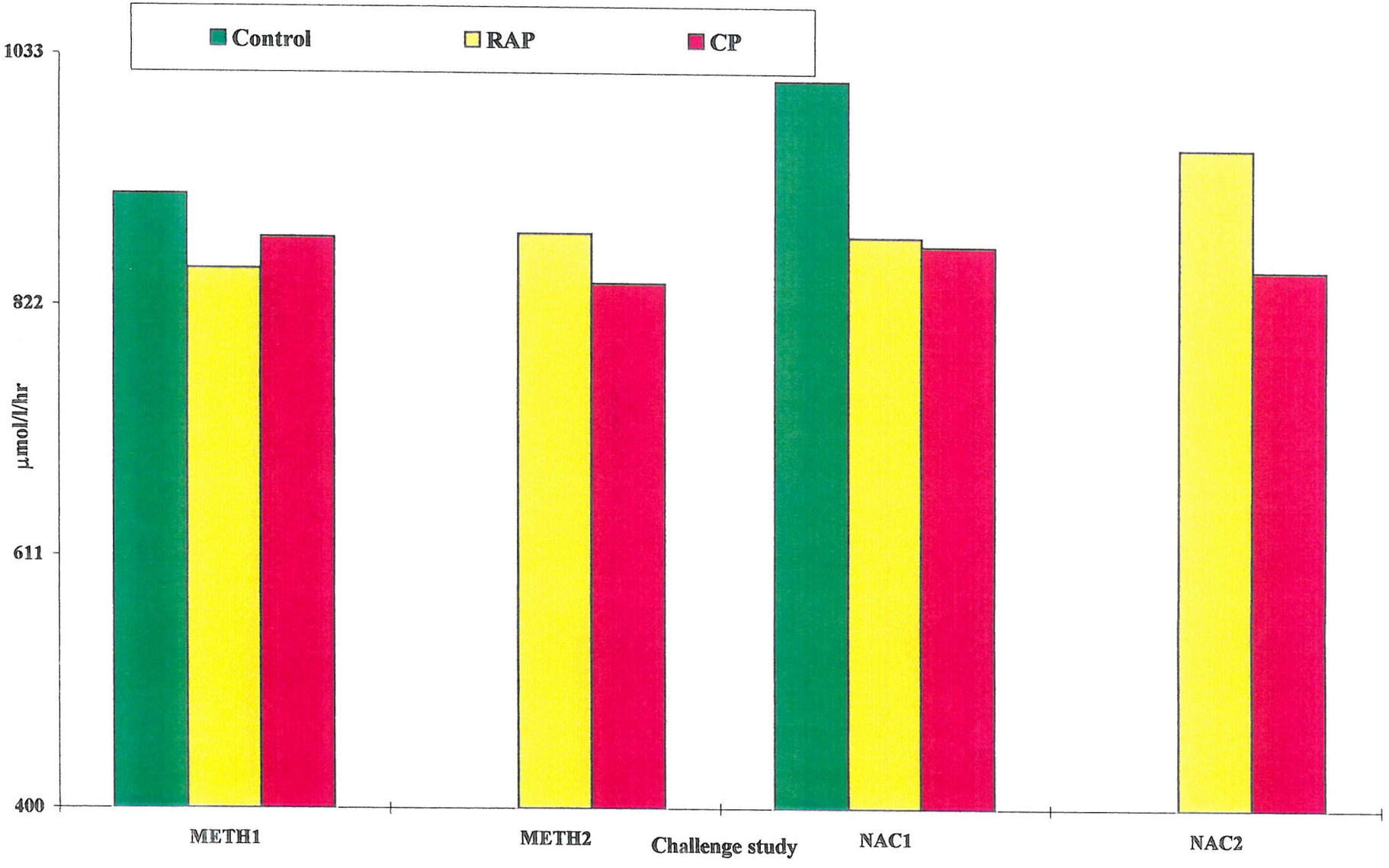


Table 4.8.3.6.6. Group mean AUC (µmol/l/hr) for plasma serine in challenge studies

4.8.3.7. Plasma Glutathione

The methodology used to analyse plasma glutathione (GSH) is described in Appendix A. It measures total GSH (GSH + GSSG). This has been refined, developed and established as routine in the Pancreato-Biliary Laboratories at Manchester Royal Infirmary (Schofield 1992 & 1993). Unfortunately, the results from a number of the specimens are not available because of haemolysis. Erythrocytes have GSH levels of ~ x 1000 that of plasma. Thus, even slight haemolysis in processing causes variations in plasma GSH that render results unreliable. Table 4.8.3.7.1. reports the percentage of plasma GSH analyses that were acceptable.

	Percent samples acceptable per challenge study			
	NAC 1	**Meth 1**	**NAC 2**	**Meth 2**
Controls	100%	100%	N/A	N/A
RAP	67%	89%	75%	75%
CP	100%	100%	33%	33%

Table 4.8.3.7.1. Percentage of acceptable plasma GSH analyses.

The plasma GSH results have therefore been analysed bearing in mind the paucity of figures available, particularly in the post AOT phase of the study.

No difference between the fasting plasma GSH level of male and female subjects was observed, Table 4.8.3.7.2. Combining genders, the average fasting plasma GSH before AOT, in controls was (mean +/- 1 s.d.), 6.06 +/- 2.48 μmol/l; in patients with RAP 4.78 +/- 1.21 and in CP 3.43 +/- 1.36, Table 4.8.3.7.2. These figures do not reach significance after the Bonferroni correction for multiple comparisons. After AOT a difference appeared between the two pancreatitis groups, RAP 4.07 +/- 0.85; CP 1.73 +/- 0.36 ($p < 0.003$), Table 4.8.3.7.2. But, this difference is due to the low n-value (n=2) of individuals with CP in whom post-

AOT results were available. If just the individuals where values both pre- and post-AOT are available then no significant change occurred (* p = 0.45), Table 4.8.3.7.2. With the above caveats borne in mind, Figures 4.8.3.7.1. - 3 plot the profiles of mean plasma glutathione in the three subject groups after oral loading with NAC and methionine. Actual values appear in Table 4.8.3.7.3. Figure 4.8.3.7.3 plots the values of the plasma GSH profiles of all patients with CP, whereas Figure 4.8.3.7.4 only plots individuals where data is available both before and after AOT (n=2). Figures 4.8.3.7.5. & 6. collate the profiles of the three subject group before and after AOT. Oral loading with either NAC or methionine did not appear to result in any demonstrable change in plasma glutathione concentration during the 9 hour observation period after dosing, Table 4.8.3.7.4.

In place of AUC analyses, the mean value across the profile of each group was calculated by averaging the mean values obtained for each time-point, Table 4.8.3.7.3. This reveals that a history of pancreatitis is associated with a lower plasma GSH concentration than healthy controls, but there is no differentiation between RAP and CP prior to AOT, Table 4.8.3.7.5. Ten weeks AOT, containing 1.6g L-methionine daily had little effect on plasma glutathione levels in any subject group. The small numbers, particularly in the CP group, precludes a robust comparison, but there was certainly no evidence that GSH rises with AOT. Indeed, if the average plasma GSH concentrations of the 2 individuals with CP who underwent all 4 challenges are examined, then the mean of phases 1 and 2; 2.32 +/- 0.56 μmol/l, is higher than those obtained in phase 3 and 4 after AOT; 1.67 +/- 0.24 μmol/l, suggesting AOT results in a fall of plasma GSH (p = 0.002). This remains significant after the Bonferroni correction.

Summary of plasma GSH results in challenge study

- Oral methionine and NAC have no discernible effect on plasma GSH profiles.
- Plasma GSH appears to be lower in RAP and CP than controls subjects, before AOT.
- AOT does not normalise plasma GSH of patients, indeed limited evidence suggests it may fall further.

Phase		Control	RAP	CP	Allpanc		t tests		
Before AOT	**means**	6.06	4.78	3.43	4.26	c v cp	0.029	c v allp1	0.089
Both genders	**sd**	2.48	1.21	1.28	1.36	c v rap	0.217		
After AOT	**means**		4.07	1.73	3.48	rap1 v cp1	0.094	* *rap2 v cp2*	* *0.453*
	sd		0.85	0.36	1.31	rap2 v cp2	0.003	c v allp2	0.025
Before AOT	**means**	6.60	4.74	3.38	4.06	c v cp	0.172	c v allp1	0.254
Male	**sd**	3.57	1.22	1.47	1.44	c v rap	0.386		
After AOT	**means**		4.38	1.47	3.65	rap1 v cp1	0.206		
	sd		1.03	N/A	1.68	rap2 v cp2	N/A	c v allp2	0.206
Before AOT	**means**	5.80	4.81	4.50	4.71	c v cp	0.351	c v allp1	0.164
Female	**sd**	0.83	1.38	1.25	1.22	c v rap	0.293		
After AOT	**means**		3.76	1.98	3.31	rap1 v cp1	0.803		
	sd		0.69	N/A	1.22	rap2 v cp2	N/A	c v allp2	0.017

t tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.694	0.938	0.416
After AOT		0.443	N/A

* *using only values where results are available both before and after AOT*

Individual group data from each phase

Plasma GSH (μmol/l)	Before AOT mean	sd	After AOT mean	sd
control nac	6.6	2.8		
control meth	5.5	2.2		
rap nac	4.8	0.7	4.7	1.5
rap meth	5.0	1.8	3.5	0.6
cp nac	4.0	1.7	1.5	0.4
cp meth	3.5	1.6	2.0	0.4

Table 4.8.3.7.2. Time Zero Plasma GSH (μmol/l) in mixed-and single gender groups (means and s.d.)

Figure 4.8.3.7.1. **Mean plasma GSH profiles: control group data (+/- 1 s.d.)**

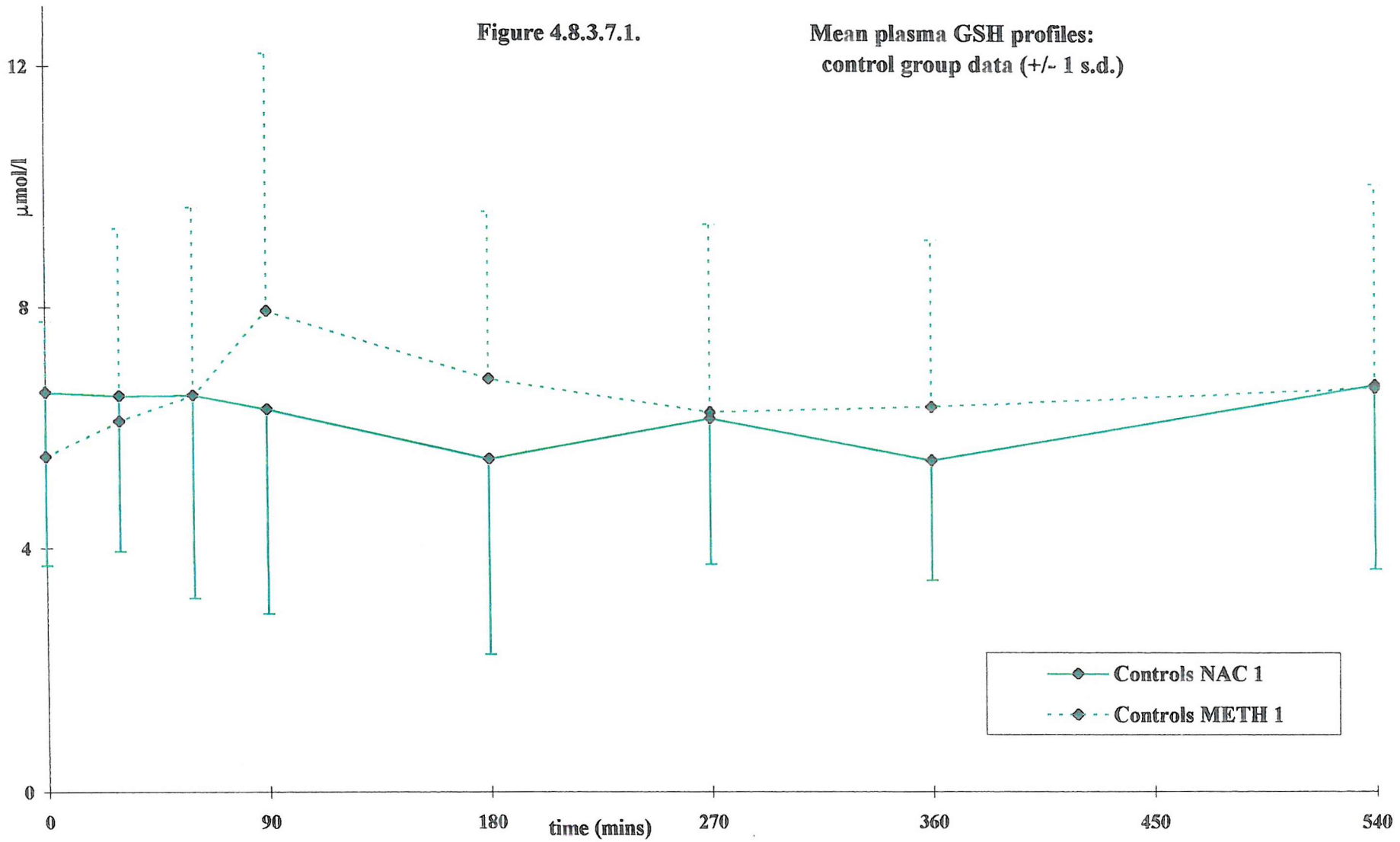


Figure 4.8.3.7.2. **Mean plasma GSH profiles: RAP group data (+/- 1sd)**

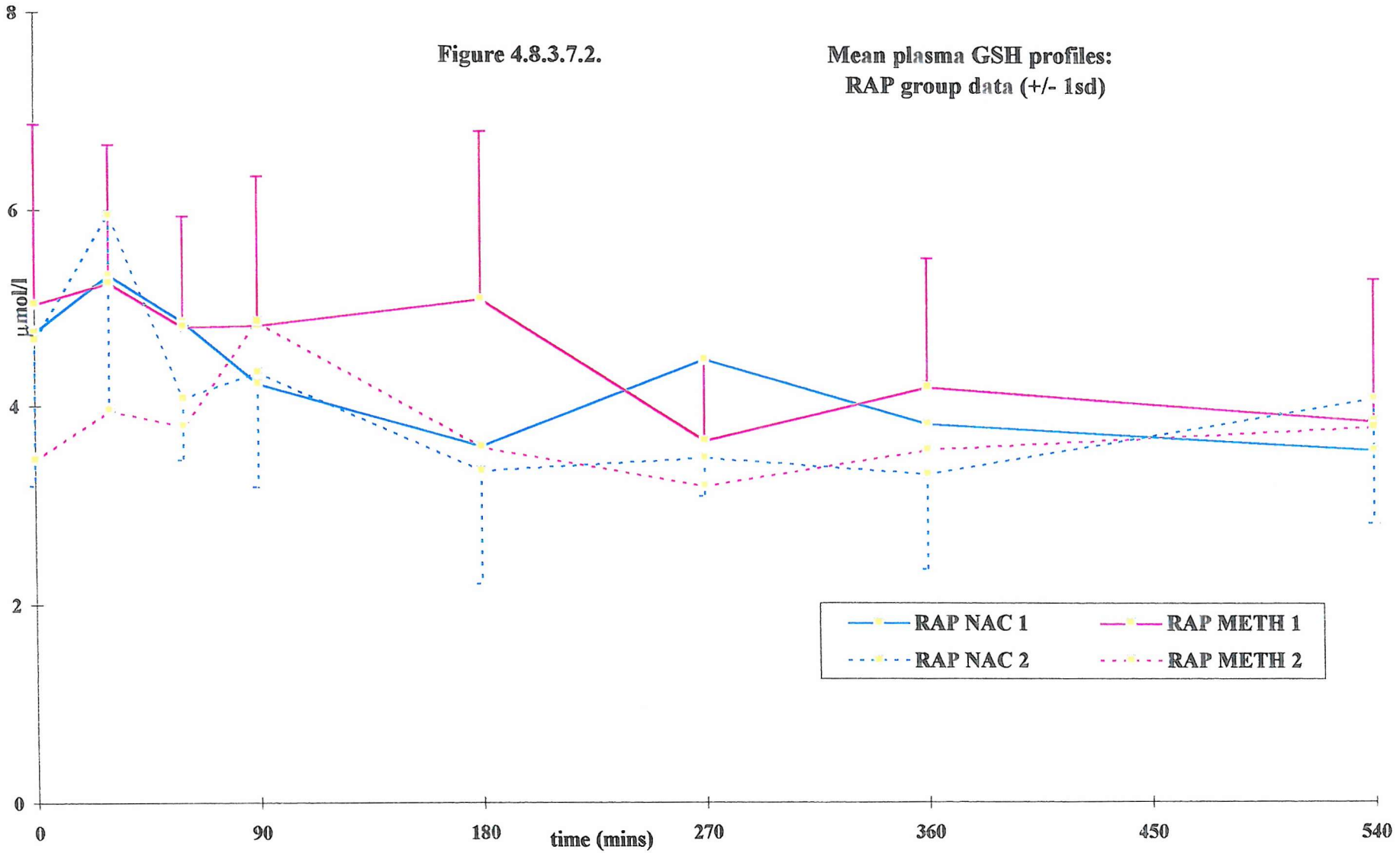


Figure 4.8.3.7.3. **Mean plasma GSH profiles: in CP of all patients analysed pre and post AOT (+/- 1 s.d.)**

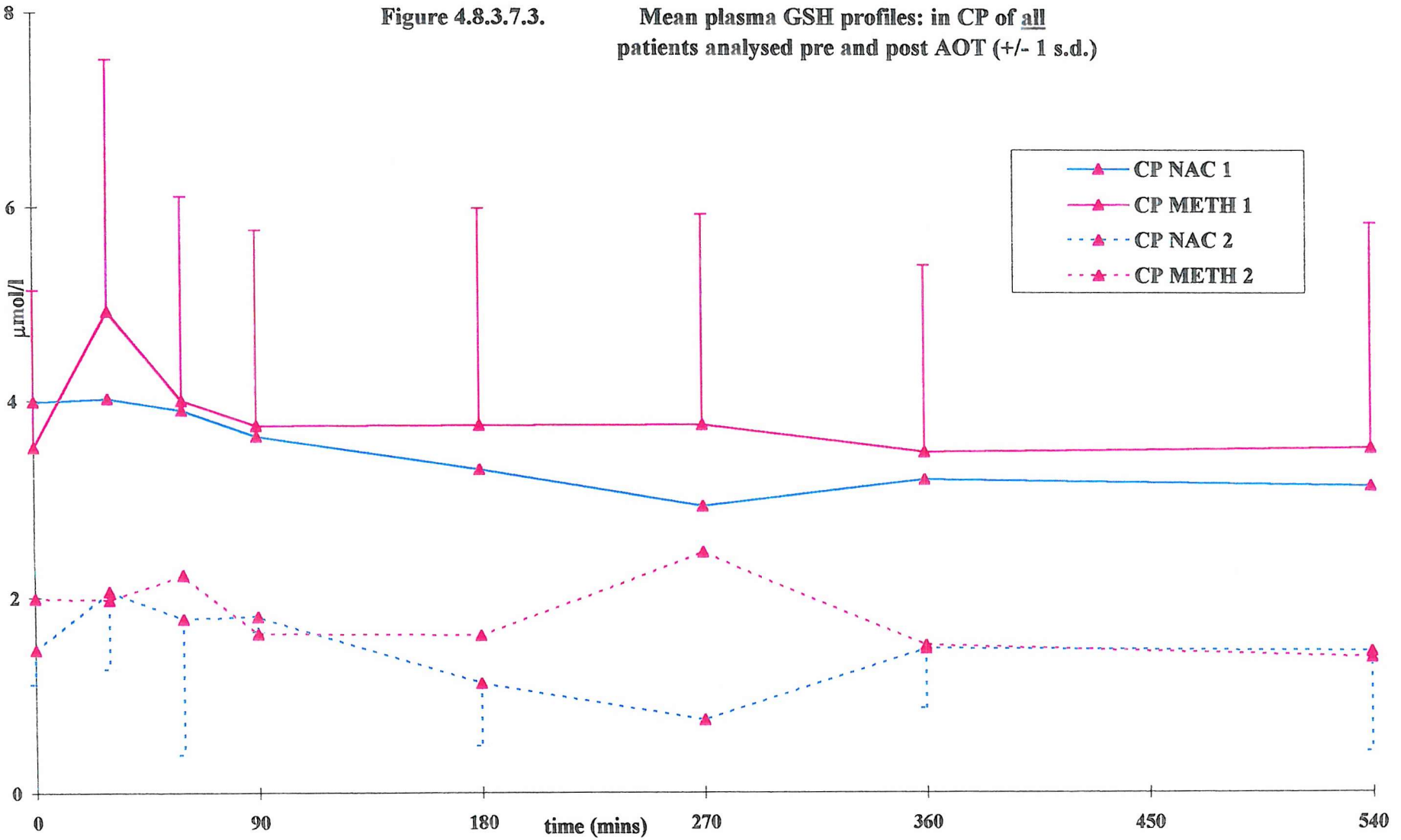


Figure 4.8.3.7.4. Mean plasma GSH profiles: in CP of only patients analysed both pre and post AOT(+/- 1 s.d.)

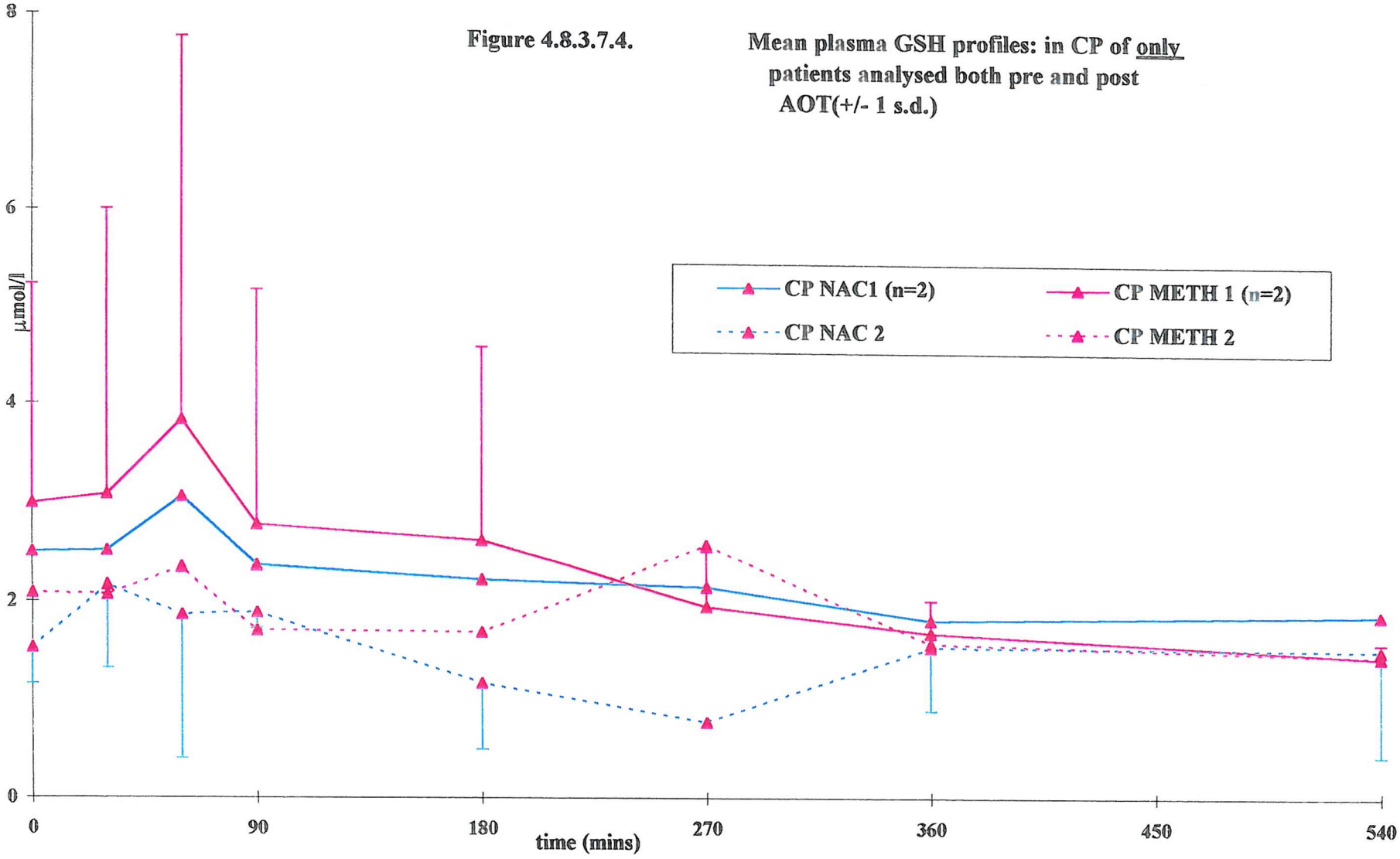


Figure 4.8.3.7.5. **Mean plasma GSH profiles: group data before AOT (+/- 1sd)**

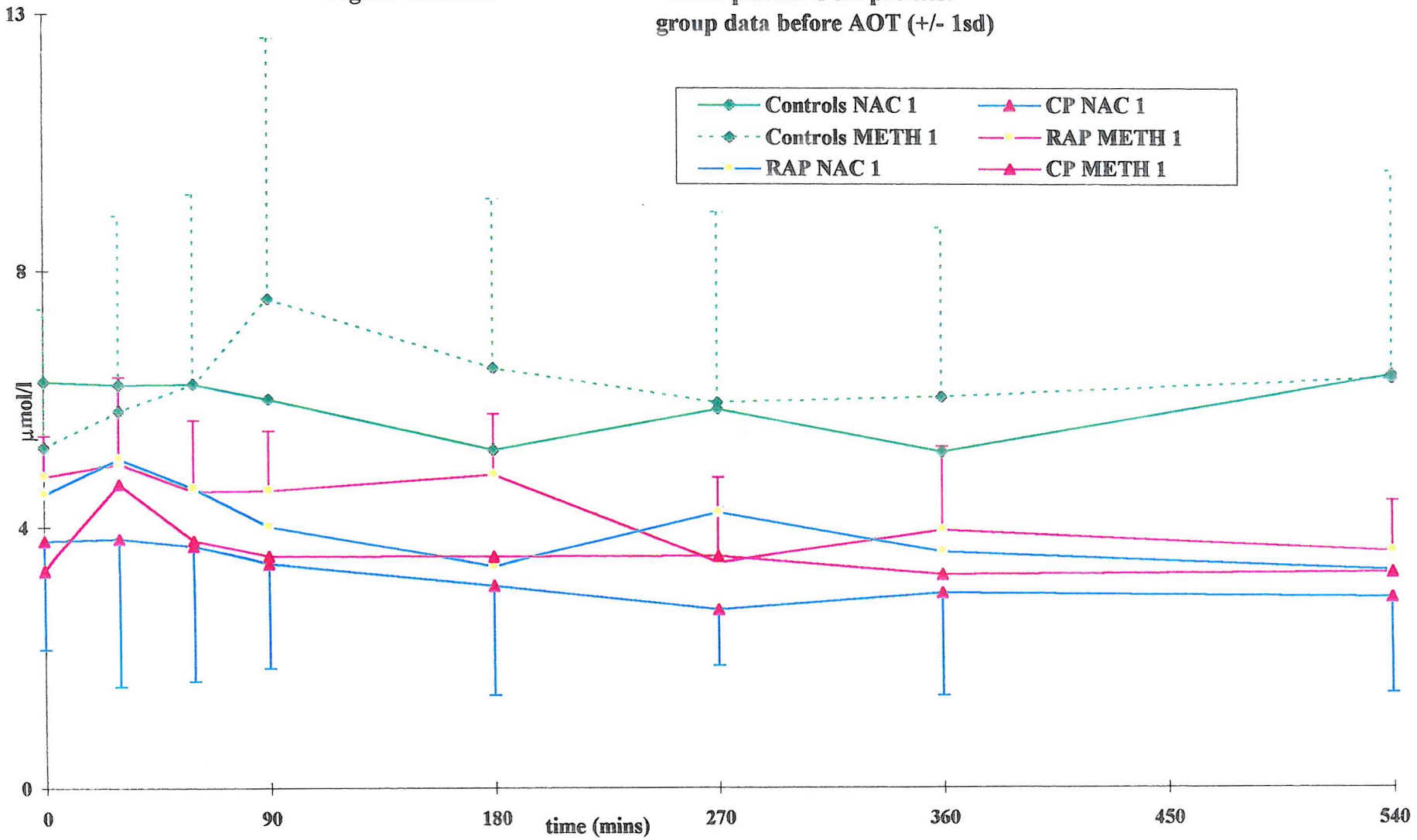
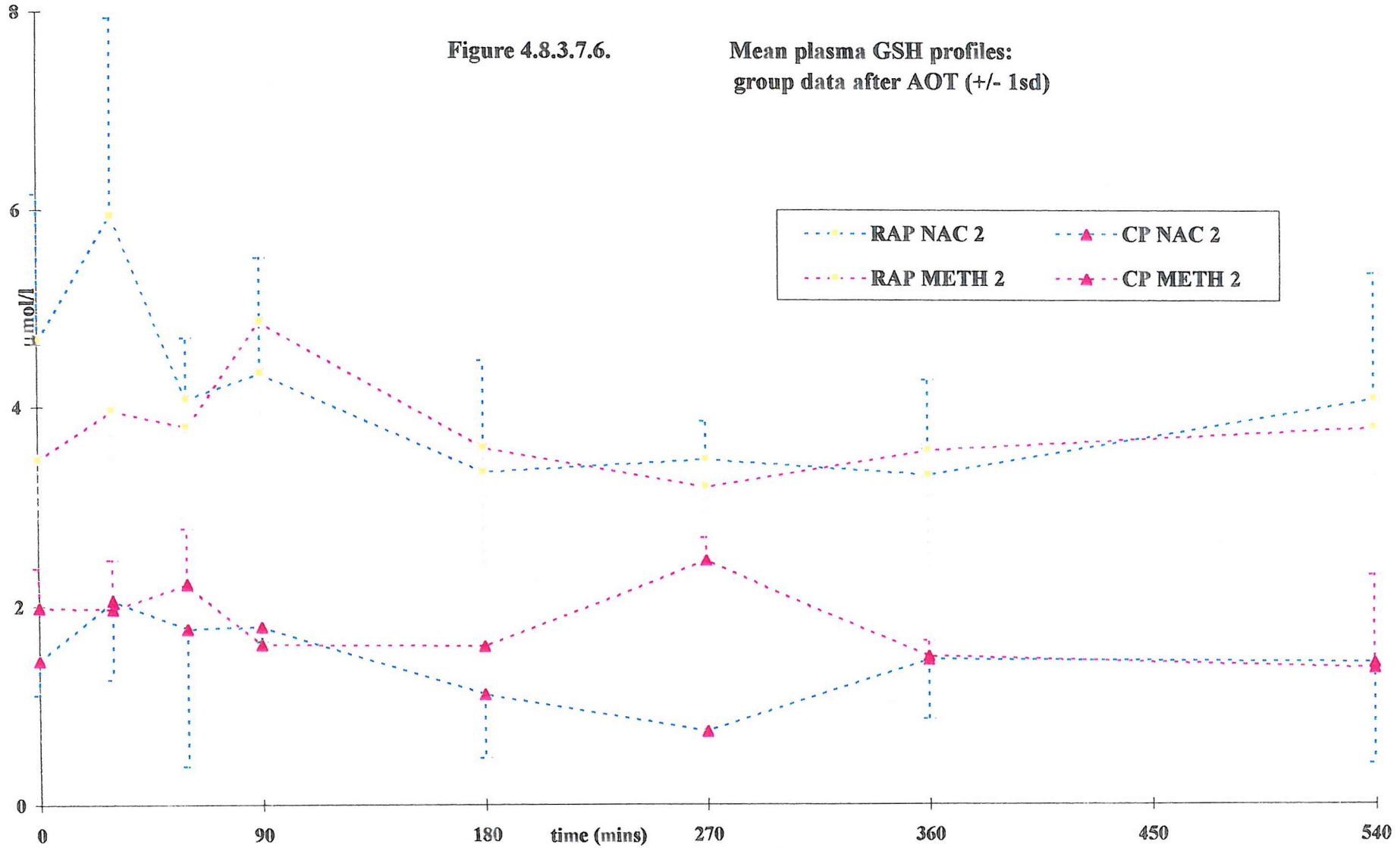


Figure 4.8.3.7.6. **Mean plasma GSH profiles: group data after AOT (+/- 1sd)**



	Time (mins)	0	30	60	90	180	270	360	540	Mean across profile
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control (n=8)	mean	5.52	6.12	6.56	7.96	6.85	6.30	6.40	6.73	6.6
	sd	2.25	3.19	3.10	4.26	2.77	3.11	2.77	3.38	0.7
RAP (n=7)	mean	5.05	5.26	4.81	4.83	5.11	3.67	4.22	3.89	4.6
	sd	1.83	1.41	1.14	1.53	1.71	0.82	1.32	1.45	0.6
CP (n=6)	mean	3.52	4.93	4.00	3.75	3.76	3.77	3.50	3.55	3.8
	sd	1.63	2.59	2.11	2.02	2.24	2.17	1.93	2.32	0.5
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP (n=5)	mean	3.46	3.97	3.81	4.88	3.60	3.21	3.59	3.83	3.8
	sd	0.59	0.66	0.92	1.20	1.18	0.71	1.20	0.59	0.5
CP (n=2)	mean	1.99	1.98	2.23	1.63	1.62	2.47	1.52	1.40	1.9
	sd	0.37	0.47	0.54	na	na	0.23	0.16	0.93	0.4
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control (n=8)	mean	6.60	6.55	6.56	6.33	5.51	6.19	5.50	6.79	6.3
	sd	2.85	2.58	3.33	3.35	3.21	2.41	1.96	3.06	0.5
RAP (n=7)	mean	4.75	5.34	4.86	4.24	3.61	4.50	3.85	3.59	4.3
	sd	0.67	1.42	1.17	0.98	1.00	1.41	1.37	0.84	0.6
CP (n=6)	mean	3.99	4.03	3.91	3.64	3.31	2.94	3.21	3.16	3.5
	sd	1.72	2.36	2.16	1.68	1.77	0.92	1.68	1.57	0.4
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP (n=6)	mean	4.68	5.95	4.08	4.35	3.35	3.49	3.33	4.12	4.2
	sd	1.49	2.00	0.62	1.17	1.13	0.39	0.97	1.28	0.9
CP (n=2)	mean	1.46	2.07	1.79	1.81	1.13	0.75	1.49	1.46	1.5
	sd	0.35	0.80	1.39	0.15	0.64	na	0.62	1.03	0.4

(n) modal number analyses at each time point

Table 4.8.3.7.3. **Group mean for plasma GSH (μmol/l) in challenge studies.**

Comparison	Time (x)						
	30	**60**	**90**	**180**	**270**	**360**	**540**
cn0 v cn(x)	0.907	0.944	0.655	0.085	0.380	0.075	0.760
cm0 v cm(x)	0.148	0.027	0.037	0.078	0.324	0.242	0.062
rn0 v rn(x)	0.036	0.840	0.236	0.117	0.669	0.352	0.111
rm0 v rm(x)	0.991	0.352	0.686	0.871	0.152	0.049	0.340
r2n0 v r2n(x)	0.209	0.336	0.684	0.177	0.078	0.061	0.547
r2m0 v r2m(x)	0.316	0.513	0.037	0.600	0.325	0.668	0.098
cpn0 v cpn(x)	0.942	0.861	0.035	0.248	0.070	0.108	0.244
cpm0 v cpm(x)	0.037	0.307	0.640	0.692	0.721	0.973	0.970
cp2n0 v cp2n(x)	0.306	0.735	0.253	0.350	N/A	0.914	1.000
cp2m0 v cp2m(x)	0.874	0.311	N/A	N/A	0.126	0.425	0.637

c - control
r - RAP pre-AOT
r2 - RAP post-AOT
cp - CP pre-AOT
cp2 - CP post-AOT

n - NAC load
m - methionine load
0 - time zero
(x) - time (x)

Table 4.8.3.7.4. **Paired t-test (p-value) of plasma GSH at each profile time-point versus baseline value**

t-tests - comparison of mean plasma GSH concentration										
	Time (minutes)	0	30	60	90	180	270	360	540	Mean across profile
meth1 v meth1	c v rap	0.651	0.513	0.175	0.112	0.157	0.050	0.075	0.056	**4.E-05**
meth1 v meth1	c v cp	0.078	0.456	0.092	0.045	0.052	0.099	0.040	0.059	**9.E-07**
meth1 v meth2	c v rap	0.037	0.103	0.043	0.109	0.025	0.027	0.028	0.046	**7.E-07**
meth1 v meth2	c v cp	**0.003**	0.008	**0.006**	na	na	0.010	**0.002**	**0.006**	**6.E-09**
meth1 vs meth1	rap v cp	0.127	0.789	0.433	0.337	0.288	0.917	0.460	0.767	1.E-02
meth2 v meth 2	rap v cp	0.026	0.025	0.056	na	na	0.131	0.008	0.135	**8.E-07**
meth1 v meth2	rap v rap	0.048	0.083	0.123	0.961	0.155	0.355	0.386	0.922	8.E-03
meth1 v meth2	cp v cp	0.076	0.039	0.109	na	na	0.203	0.053	0.120	**9.E-06**
NAC1 vs NAC1	c v rap	0.115	0.279	0.208	0.138	0.147	0.140	0.073	0.021	**1.E-05**
NAC1 vs NAC1	c v cp	0.056	0.083	0.097	0.076	0.130	0.011	0.037	0.015	**1.E-08**
NAC1 v NAC2	c v rap	0.131	0.633	0.075	0.157	0.115	0.025	0.020	0.051	**1.E-04**
NAC1 v NAC2	c v cp	**0.001**	**0.005**	0.030	0.007	0.007	na	**0.002**	**0.006**	**1.E-11**
NAC1 vs NAC1	rap v cp	0.349	0.268	0.358	0.501	0.719	0.037	0.470	0.566	1.E-02
NAC2 v NAC 2	rap v cp	**0.003**	0.011	0.241	**0.003**	0.035	na	0.053	0.089	**1.E-05**
NAC1 v NAC2	rap v rap	0.915	0.550	0.134	0.867	0.689	0.114	0.428	0.410	4.E-01
NAC1 v NAC2	cp v cp	0.015	0.133	0.211	0.044	0.047	na	0.082	0.185	**2.E-07**

meth 1 - methionine load pre-AOT
meth 2 - methionine load post-AOT

NAC 1 -NAC load pre-AOT
NAC 2 - NAC load post-AOT

c - Controls
rap - Recurrent acute pancreatitis
cp - Chronic pancreatitis

Table 4.8.3.7.5. **Students' t-tests - comparison of mean plasma GSH concentration**

4.8.3.8. Whole Blood Glutathione

The problems with haemolysis affecting quantitative analysis of whole blood glutathione (wbGSH) were not as common as that experienced with plasma GSH. However, 12 of the 76 individual profiles (16%) had precipitated haemoglobin, and the low results recorded as a result of this, are likely to be erroneous (Appendix C). These results have therefore been omitted from further analysis.

Gender sub-division demonstrated there to be no difference between males and females of the three groups, Table 4.8.3.8.1. The mixed gender mean wbGSH baseline values were also similar. Following 10 weeks AOT, fasting wbGSH remained unchanged in both groups of patients with pancreatitis.

Figures 4.8.3.8.1. - 3. plot the profiles of the controls and patients with pancreatitis in the challenge studies. Neither NAC nor methionine resulted in any change in wbGSH concentration over the nine hour observation period, irrespective of antioxidant status, Tables 4.8.3.8.2. - 3. The mean wbGSH concentration of patients with CP after AOT fell 90 minutes after oral loading. No other time point within that or any other profiles altered compared to baseline, and it is unlikely that this finding is true.

Table 4.8.3.8.4. shows that at no time point did any of the studies groups differ from one another. As there was no change in wbGSH concentration throughout the challenge profiles, an average of the group means at each time point of each phase, was calculated, Table 4.8.3.8.5. These suggest that patients with RAP (*) had lower wbGSH than controls in the methionine challenge study before AOT ($p < 0.0001$), Figure 4.8.3.8.4. No difference persisted after AOT and no other inter-group comparison revealed any differences.

Average (s.d.) **wbGSH** (μmol/g Hb)	Control	RAP	CP
METH1	8.85 (0.15)	8.14 (0.17) *	8.69 (0.20)
METH2		8.77 (0.19)	9.21 (0.39)
NAC1	8.87 (0.12)	9.00 (0.20)	9.11 (0.38)
NAC2		8.67 (0.29)	8.80 (0.19)

* *p = 4.E-07 vs. control group.*

Table 4.8.3.8.5. Average wbGSH (mmol/g Hb) of the group means at each time point of each study phase.

Summary of whole blood GSH results in challenge study

- No difference in baseline wbGSH was seen between controls and patients with a history of pancreatitis.
- AOT did not produce any discernible effect on baseline wbGSH profiles.
- Oral loading with NAC and methionine failed to produce an effect on wbGSH profiles irrespective of antioxidant status.

Phase		Control	RAP	CP	Allpanc	t tests			
Before AOT	**means**	9.10	8.21	8.41	8.29	c v cp	0.490	c v allp1	0.291
Both genders	**sd**	1.83	0.93	1.73	1.25	c v rap	0.246		
After AOT	**means**		8.61	8.99	8.77	rap1 v cp1	0.803		
	sd		0.77	1.07	0.89	rap2 v cp2	0.483	c v allp2	0.651
Before AOT	**means**	8.19	6.07	7.95	6.90	c v cp	0.817	c v allp1	0.197
Male	**sd**	1.43	2.16	1.59	2.07	c v rap	0.110		
After AOT	**means**		8.58	8.65	8.61	rap1 v cp1	0.177		
	sd		0.99	1.20	1.01	rap2 v cp2	0.923	c v allp2	0.585
Before AOT	**means**	10.60	8.28	9.35	8.64	c v cp	0.564	c v allp1	0.128
Female	**sd**	1.46	1.09	2.17	1.40	c v rap	0.090		
After AOT	**means**		8.67	9.67	9.07	rap1 v cp1	0.612		
	sd		0.35	0.23	1.40	rap2 v cp2	0.031	c v allp2	0.204

t tests	c(m) v c(f)	rap(m) v rap(f)	cp(m) vs cp(f)
Before AOT	0.082	0.092	0.523
After AOT		0.854	0.190

Table 4.8.3.8.1. Combined mean baseline whole blood GSH (μmol/gHb) in mixed and single gender groups

Figure 4.8.3.8.1. Mean wbGSH (µmol/g Hb) profiles: Controls

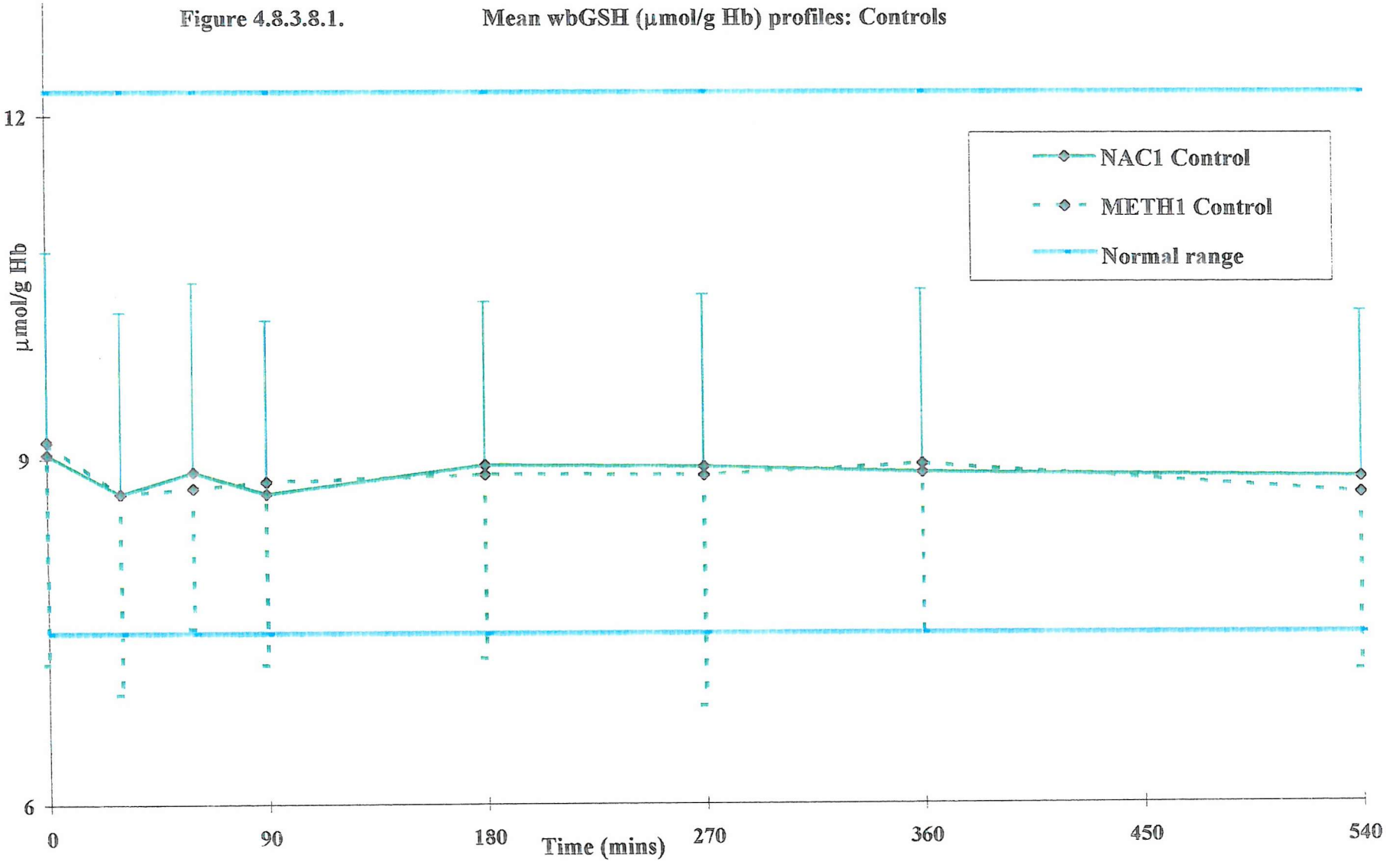


Figure 4.8.3.8.2. Mean wbGSH (μmol/g Hb) profiles: RAP

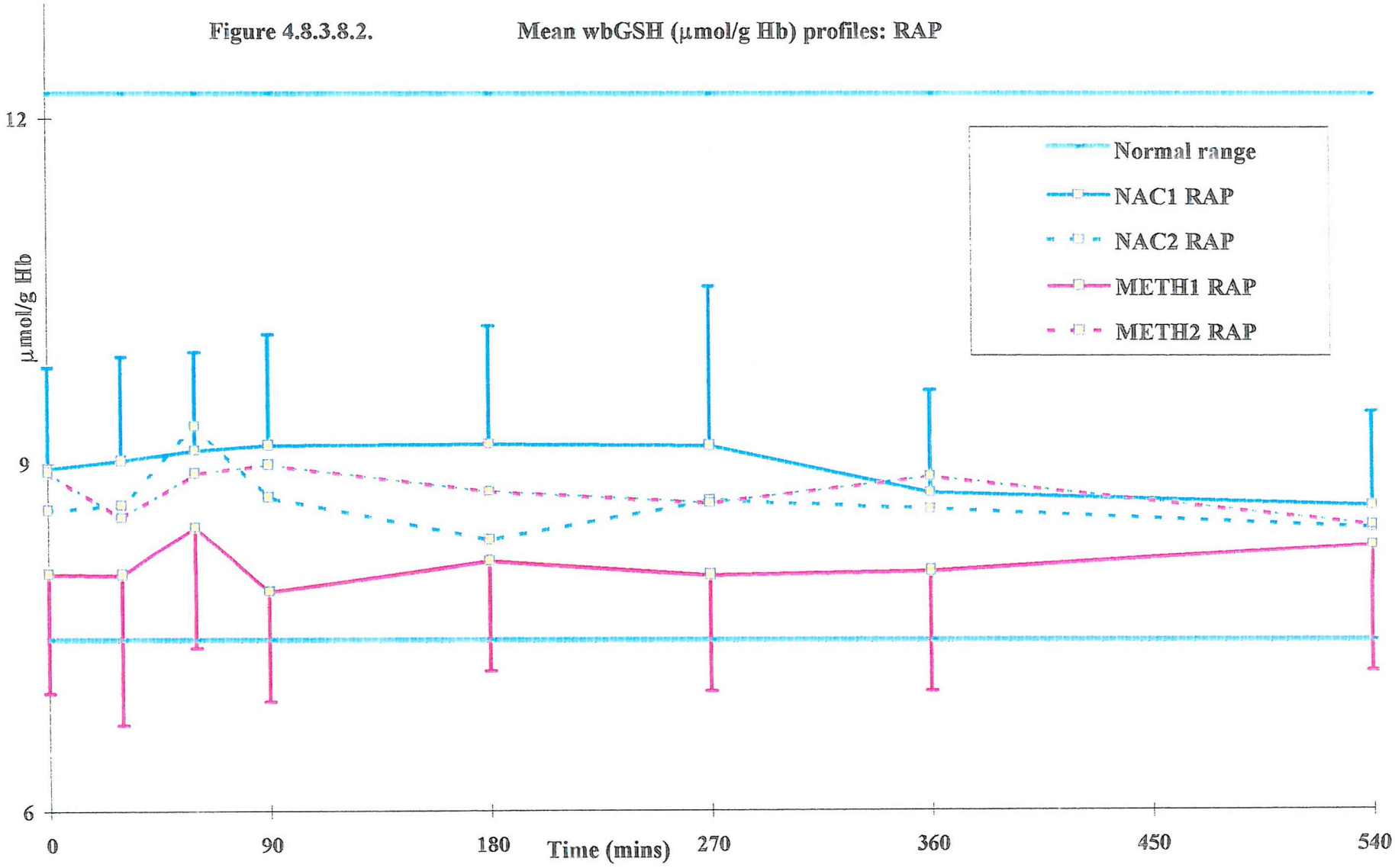
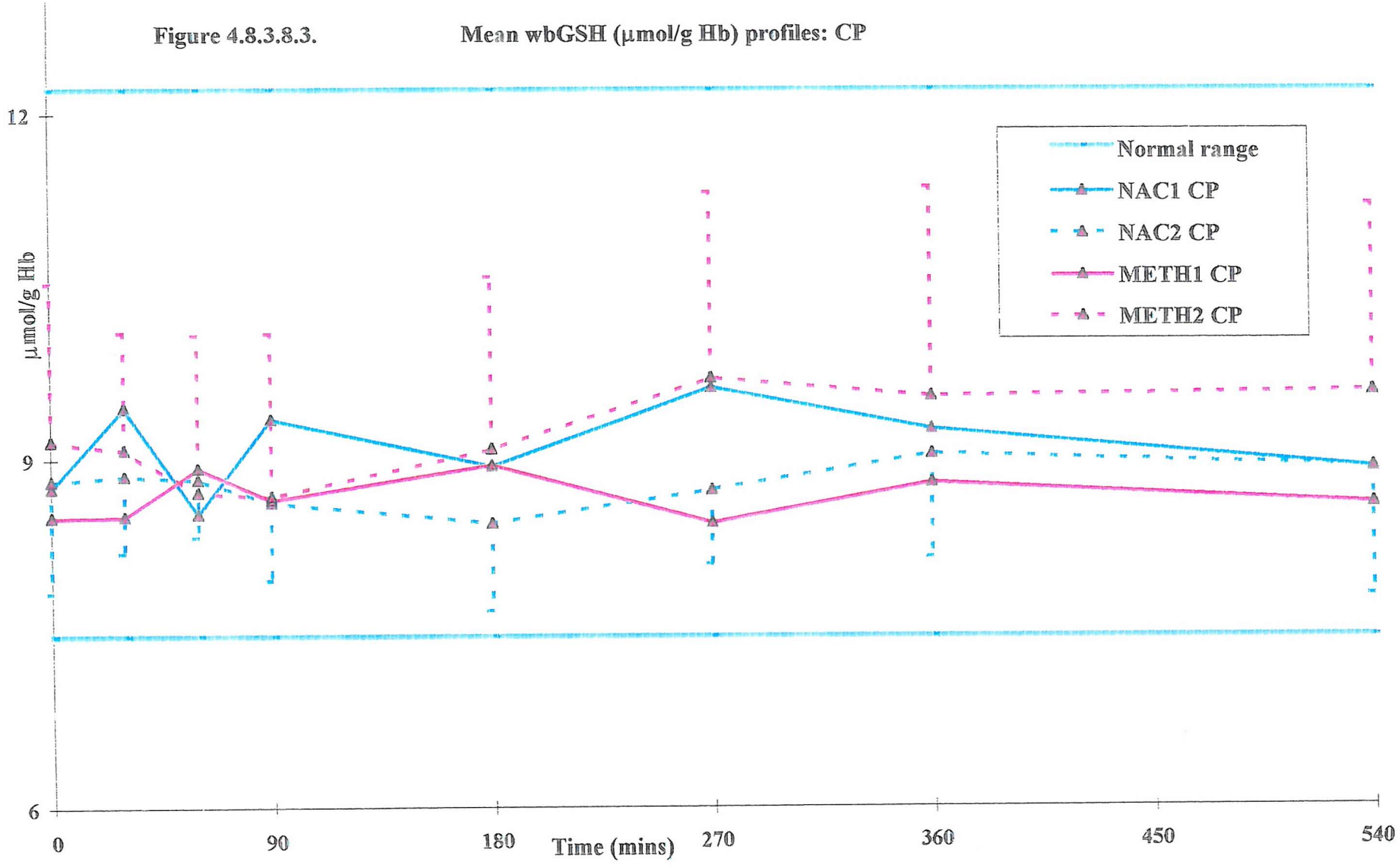


Figure 4.8.3.8.3. Mean wbGSH (µmol/g Hb) profiles: CP



	Time (mins)	0	30	60	90	180	270	360	540
	Before AOT	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**	**METH 1**
Control	mean	9.15	8.70	8.75	8.80	8.87	8.87	8.97	8.72
	sd	1.92	1.73	1.21	1.58	1.59	2.01	1.47	1.55
RAP	mean	8.06	8.05	8.45	7.91	8.17	8.05	8.09	8.31
	sd	1.03	1.30	1.02	0.95	0.94	1.00	1.04	1.08
CP	mean	8.48	8.51	8.70	8.50	8.87	8.38	8.77	8.54
	sd	1.44	1.54	1.71	1.49	1.78	1.46	1.36	1.54
	After AOT	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**	**METH 2**
RAP	mean	8.22	7.80	8.16	8.15	8.03	7.85	8.08	7.94
	sd	1.41	1.37	1.58	1.75	1.47	1.54	1.65	1.08
CP	mean	9.16	9.09	8.72	8.69	9.10	9.72	9.56	9.61
	sd	1.37	1.02	1.36	1.41	1.49	1.61	1.81	1.62
	Before AOT	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**	**NAC 1**
Control	mean	9.04	8.70	8.89	8.70	8.96	8.94	8.89	8.85
	sd	1.78	1.59	1.66	1.52	1.44	1.51	1.60	1.46
RAP	mean	8.96	9.03	9.12	9.16	9.18	9.16	8.76	8.65
	sd	0.88	0.91	0.86	0.96	1.03	1.39	0.90	0.82
CP	mean	7.74	8.16	7.21	7.90	7.68	7.95	8.01	8.09
	sd	2.32	2.43	2.10	2.48	2.32	2.64	2.22	2.20
	After AOT	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**	**NAC 2**
RAP	mean	7.66	7.65	8.29	7.73	7.42	7.94	7.79	7.58
	sd	1.94	2.08	2.35	2.03	1.87	1.82	1.87	1.82
CP	mean	8.82	8.87	8.84	8.64	8.46	8.75	9.06	8.95
	sd	0.96	0.67	0.50	0.67	0.75	0.65	0.90	1.11

Table 4.8.3.8.2. **Group mean whole blood GSH (μmol/l) in challenge study profiles**

Comparison	Time (x)						
	30	**60**	**90**	**180**	**270**	**360**	**540**
cn0 v cn(x)	0.025	0.178	0.052	0.669	0.582	0.356	0.342
cm0 v cm(x)	0.156	0.189	0.206	0.270	0.507	0.384	0.111
rn0 v rn(x)	0.588	0.207	0.223	0.591	0.679	0.346	0.468
rm0 v rm(x)	0.990	0.250	0.552	0.583	0.993	0.434	0.204
r2n0 v r2n(x)	0.498	0.252	0.551	0.105	0.629	0.098	0.405
r2m0 v r2m(x)	0.077	0.981	0.818	0.601	0.249	0.894	0.046
cpn0 v cpn(x)	0.127	0.384	0.032	0.608	0.166	0.956	0.745
cpm0 v cpm(x)	0.156	0.134	0.500	0.404	0.890	0.915	0.831
cp2n0 v cp2n(x)	0.845	0.973	0.444	0.119	0.759	0.490	0.548
cp2m0 v cp2m(x)	0.862	0.371	**0.003**	0.544	0.343	0.593	0.564

c - control
r - RAP pre-AOT
r2 - RAP post-AOT
cp - CP pre-AOT
cp2 - CP post-AOT

n - NAC load
m - methionine load
0 - time zero
(x) - time (x)

Table 4.8.3.8.3. Paired t-test of wb GSH at each profile time-point versus baseline value

t-tests (p-value) - comparison of mean whole blood GSH concentrations									
	Time (minutes)	0	30	60	90	180	270	360	540
meth1 v meth1	c v rap	0.180	0.406	0.597	0.220	0.296	0.323	0.181	0.546
meth1 v meth1	c v cp	0.505	0.833	0.829	0.872	0.926	0.682	0.848	0.927
meth1 v meth2	c v rap	0.762	0.797	0.722	0.776	0.866	0.783	0.898	0.691
meth1 v meth2	c v cp	0.990	0.602	0.975	0.898	0.798	0.398	0.530	0.323
meth1 vs meth1	rap v cp	0.555	0.536	0.561	0.329	0.379	0.585	0.324	0.683
meth2 v meth 2	rap v cp	0.709	0.237	0.770	0.672	0.656	0.171	0.420	0.153
meth1 v meth2	rap v rap	0.052	0.303	0.265	0.021	0.163	0.107	0.059	0.663
meth1 v meth2	cp v cp	0.460	0.436	0.828	0.974	0.897	0.204	0.454	0.329
NAC1 vs NAC1	c v rap	0.911	0.643	0.752	0.516	0.753	0.795	0.856	0.751
NAC1 vs NAC1	c v cp	0.837	0.525	0.772	0.588	0.998	0.553	0.715	0.929
NAC1 v NAC2	c v rap	0.566	0.939	0.627	0.990	0.351	0.744	0.736	0.553
NAC1 v NAC2	c v cp	0.767	0.794	0.933	0.917	0.423	0.747	0.813	0.893
NAC1 vs NAC1	rap v cp	0.878	0.702	0.621	0.865	0.838	0.693	0.593	0.752
NAC2 v NAC 2	rap v cp	0.708	0.690	0.499	0.889	0.811	0.925	0.497	0.434
NAC1 v NAC2	rap v rap	0.547	0.548	0.788	0.475	0.196	0.582	0.835	0.733
NAC1 v NAC2	cp v cp	0.962	0.590	0.788	0.530	0.642	0.424	0.820	0.995

meth 1 - methionine load pre-AOT
meth 2 - methionine load post-AOT

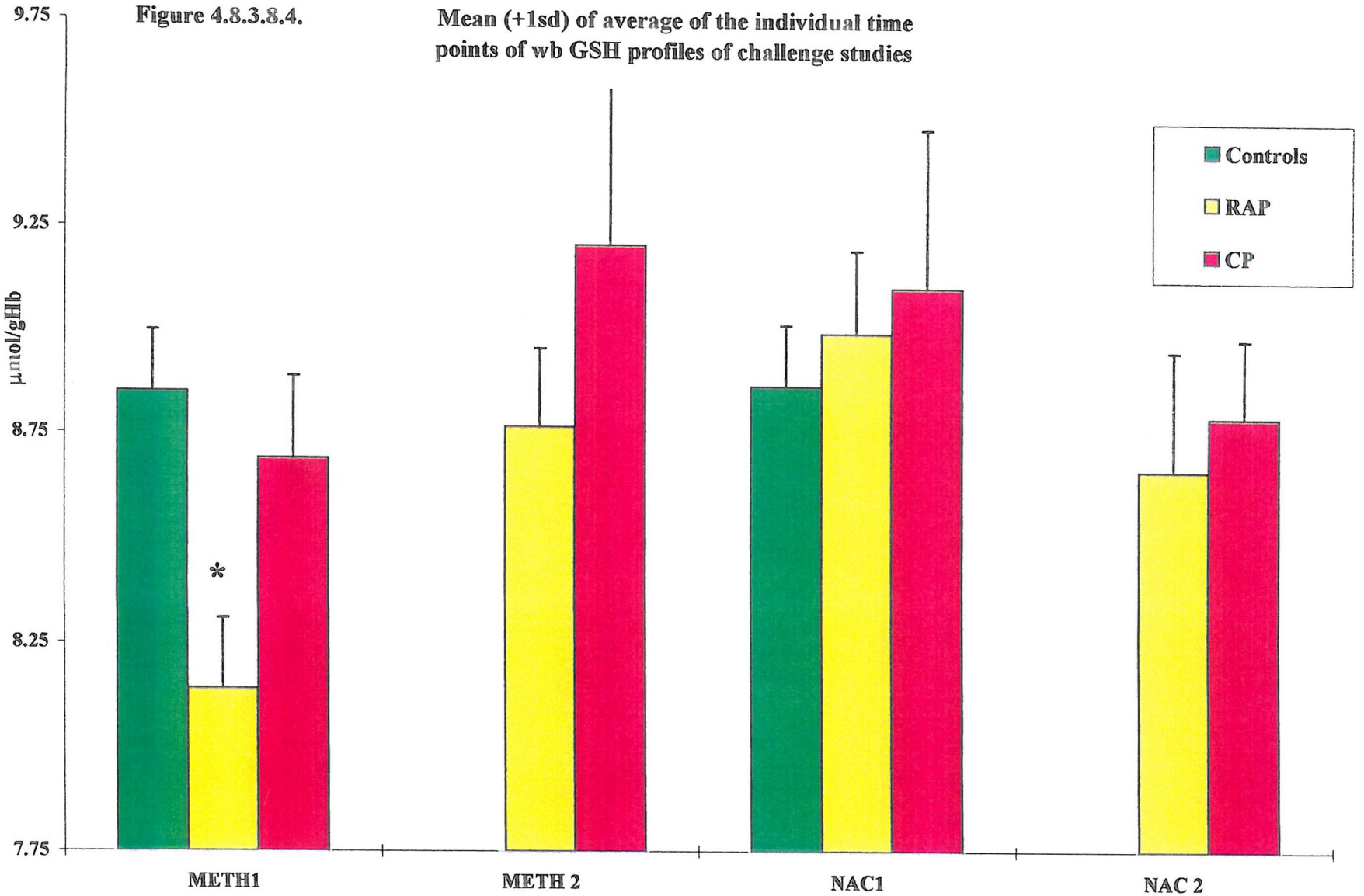
NAC 1 -NAC load pre-AOT
NAC 2 - NAC load post-AOT

c - Controls
rap - Recurrent acute pancreatitis
cp - Chronic pancreatitis

Table 4.8.3.8.4. **Students t-tests (p-value) comparisons of mean whole blood GSH concentration**

Figure 4.8.3.8.4.

Mean (+1sd) of average of the individual time points of wb GSH profiles of challenge studies



4.8.3.9. Urinary inorganic sulphate excretion

The results of the group mean 24 hour urine collections for inorganic sulphate output in the two collection periods for each phase of the study appear in Table 4.8.3.9.1. The total iSO_4 for the second 24 hour period (24 ii), i.e. that collected after oral loading, do not always correspond to the sum of the 3 shorter periods, that make up the total, because a few subjects did not provide the final 12 - 24 hour collections. By averaging the iSO_4 output of each individual from the two 24 hour collections performed before the studies phases prior to AOT, a mean baseline value was obtained, METH + NAC in Table 4.8.3.9.1. and Figure 4.8.3.9.1. Patients with chronic pancreatitis tended to excrete less than the other groups, but this did not reach statistical significance.

The statistical analyses in Table 4.8.3.9.1. reveal that the time period during which the majority of the sulphur content of the oral loading substance is excreted differs between NAC and Methionine. During the first six hours after oral NAC, controls (14.18mM) and (RAP 14.01mM) excreted significantly more iSO_4 than the same groups after methionine loading (8.76mM, $p = 0.002$) and (7.23mM, $p = 0.001$) respectively. An inverse situation was seen during the later collection periods, when the methionine-derived iSO_4 excretion peaked. Between 6 and 12 hours the iSO_4 excretion of the CP patients following oral methionine was 8.80mM compared to 5.91mM after NAC ($p = 0.008$) and in the 12 to 24 hour time period, patients with RAP differed similarly ($p = 0.003$), Table 4.8.3.9.1.

These differences are further demonstrated if the group data is combined. Before antioxidant supplementation, there was no difference in the total urinary iSO_4 excreted by the three groups in the 24 hour period after oral loading. Therefore, the results for the three groups in the NAC challenges were combined, as were the values from the methionine challenges. Urinary iSO_4 excretion was higher during the six hour time period immediately after NAC (12.64mM) than methionine (7.25mM, $p = 3.E\text{-}05$). Following methionine loading iSO_4 output peaked between

6 and 12 hours (10.64mM), compared to NAC (7.91mM, p = 4.E-07), and this difference persisted during the 12 - 24 period, Table 4.8.3.9.2.

Figure 4.8.3.9.2. (RAP2 and CP2), suggest that after ten weeks AOT, iSO_4 output might increase over their pre-AOT levels. Averaging the urinary iSO_4 output values of each individual for the 24 hour collection periods (24i) before oral NAC and methionine, the groups did not in fact differ, 17.81 vs. 18.69 mmoles (p = 0.865), Table 4.8.3.9.2. Paired t-test, Table 4.8.3.9.2, for the values obtained before and after AOT also did not differ, (RAP p = 0.959 and CP p = 0.163).

In the steady state, total body sulphur should be stable. This assumes that no prior deficiency is present and natural losses are replaced by an adequate dietary intake. In this situation total daily urinary iSO_4 output should remain constant. Any increase in total output during the 24 hours after an oral load represents iSO_4 derived from the sulphur moiety of the challenge compound. Table 4.8.3.9.3. reports the total output of iSO_4 during the two 24 hour collection periods associated with the challenge studies before and after AOT. The percentage sulphur recovered from that administered, during the first 24 hours after loading has been calculated. 81.3% of methionine-derived sulphur but only 51.3% NAC-derived sulphur was recovered from the control group (p = 0.028). The differences in sulphur recovery observed in the pancreatitis groups, also did not reach significance, irrespective of antioxidant status.

Summary of urinary inorganic sulphate output in challenge studies.

- Urinary iSO_4 output from patients with quiescent CP is low, but is not different to that of healthy controls or subjects with RAP.
- Following an oral load of NAC $UiSO_4$ output peaks by 6 hours.
- After an oral methionine load this peak is more delayed.
- Over 50% of the sulphur content of an oral load of NAC or methionine, is excreted as $UiSO_4$.

		Urine collection period (hours)				
Mean $UiSO_4$ (mmoles)		**24 i**	**0 to 6**	**6 to 12**	**12 to 24**	**24 ii**
METH1	Controls	16.73	8.76	12.14	15.39	35.67
	RAP1	15.50	7.23	10.59	15.23	30.24
	CP1	12.70	5.31	8.80	10.48	24.33
METH2	RAP2	21.92	9.86	13.33	13.64	36.84
	CP2	23.93	11.69	11.13	9.76	32.58
NAC1	Controls	18.25	14.18	8.50	9.88	31.38
	RAP1	19.34	14.01	8.51	7.19	29.71
	CP1	10.85	9.31	5.93	6.39	20.71
NAC2	RAP2	21.92	14.77	9.10	9.61	33.50
	CP2	17.44	14.11	7.73	8.70	30.54

t-test calculations	**Methionine v NAC loading studies**			
(p-value)	**0 to 6**	**6 to 12**	**12 to 24**	**24ii vs. 24ii**
Controls	**0.002**	0.016	0.047	0.321
RAP 1	**0.001**	0.137	**0.003**	0.799
CP 1	0.045	**0.008**	0.036	0.282
RAP 2	0.047	0.010	0.094	0.290
CP 2	0.548	0.155	0.575	0.572

24i - 24 hour collection period prior to loading
METH 1 - Methionine challenge prior to AOT
METH 2 - Methionine challenge after AOT

24ii - 24 hour collection period after loading
NAC 1 - NAC challenge before AOT
NAC 2 - NAC challenge after AOT

Table 4.8.3.9.1. Group mean urinary iSO_4 output (mmoles) and t-test comparisons (p-values) in challenge studies.

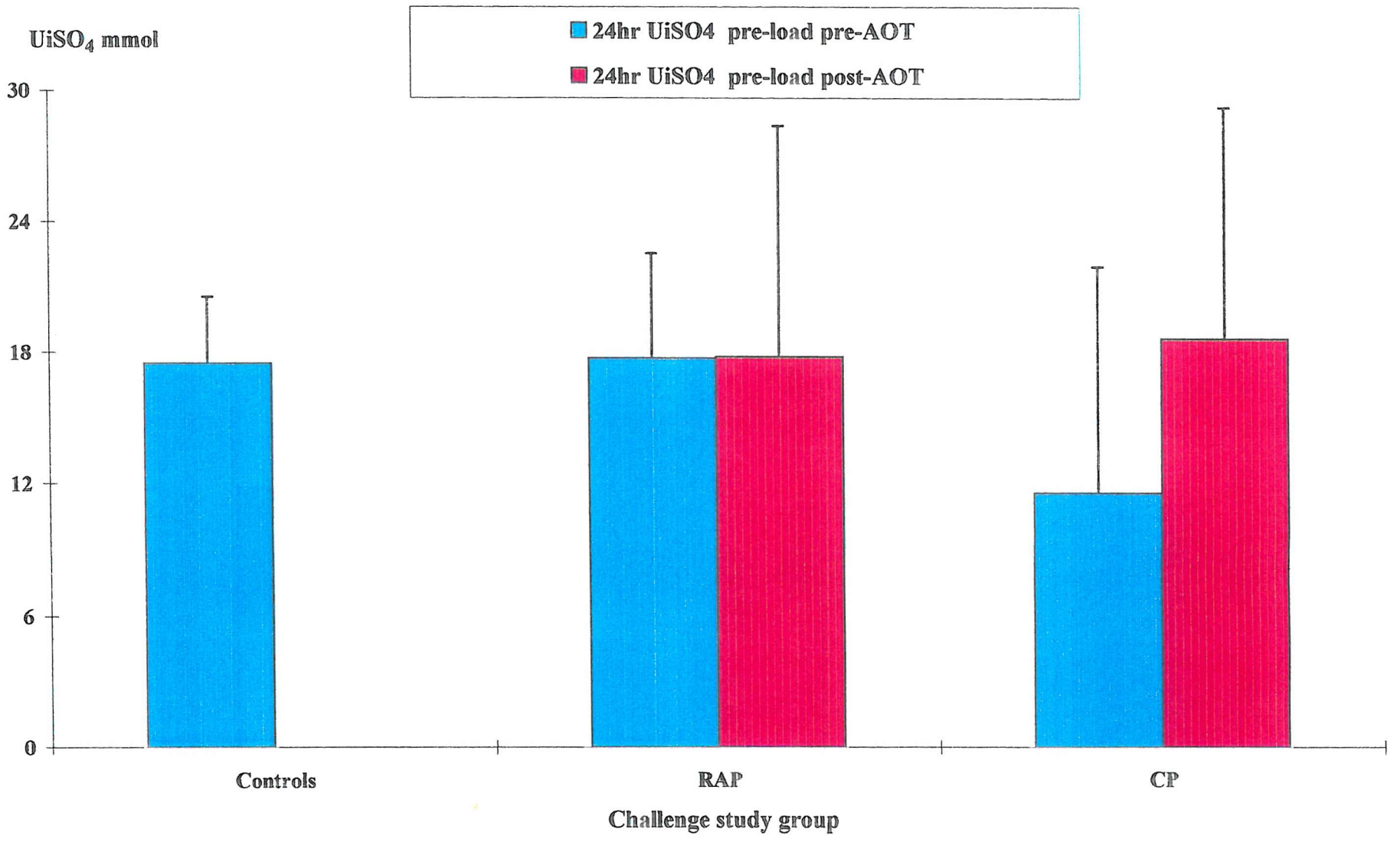


Figure 4.8.3.9.1. Group mean (+1sd) 24 hour urinary inorganic sulphate excretion of combined pre-AOT studies (mmoles)

		Group mean		Comparison	t-test results 24i vs. 24i
METH1 + NAC1	**Controls**	17.49	**METH1 + NAC1**	Control v RAP	0.896
	RAP	17.74		Control v CP	0.230
	CP	11.6		CP v RAP	0.221
METH2 + NAC2	**RAP**	17.81	**(METH1 + NAC1) /**	RAP1 v RAP2 (pr'ed)	0.959
	CP	18.69	**(METH2 + NAC2)**	CP1 v CP2 (pr'ed)	0.163

pr'ed - paired t-test

	t-test calculations	24i vs. 24i	0 to 6	6 to 12	12 to 24	24ii vs. 24ii
Mean of groups pre-AOT after Methionine		16.72	7.250	10.640	13.950	30.640
Mean of groups pre-AOTafter NAC		15.23	12.640	7.910	7.730	27.420
Paired t-test methionine vs. NAC		2.E-01	**3.E-05**	**4.E-07**	**6.E-05**	7.E-02

24i - 24 hour collection period prior to loading

RAP 1 - RAP patients prior to AOT

METH 1 - Methionine challenge prior to AOT

METH 2 - Methionine challenge after AOT

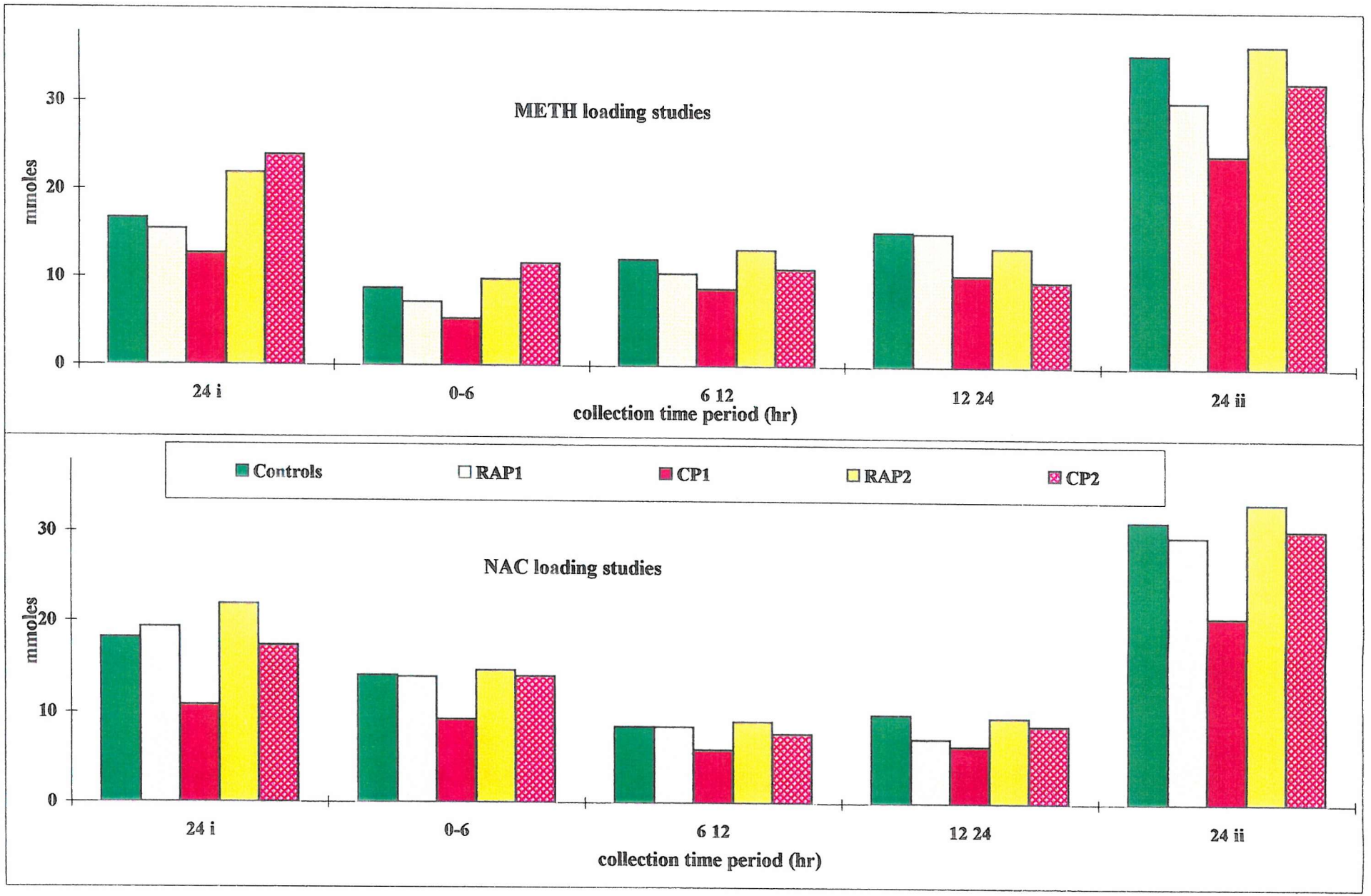
24ii - 24 hour collection period after loading

CP 1 - CP patients before AOT

NAC 1 - NAC challenge before AOT

NAC 2 - NAC challenge after AOT

Table 4.8.3.9.2. **Combined group mean urinary iSO_4 output (mmoles) and t-test p-values in challenge studies.**



Urinary iSO $_4$ output (mmoles) in challenge studies.

Figure 4.8.3.9.2. 24i - 24hr collecton pre-AOT 24ii - 24hr collection post-AOT

	Dose administered in studies		Before AOT				After AOT			
			NAC	METH	% Recovery		NAC2	METH2	% Recovery	
	mmoles NAC	mmoles Meth	24 ii-i	24 ii-i	NAC	METH	24 ii-i	24 ii-i	NAC	METH
NST	23.7	26.0								
GKY	23.6	25.8	15.3	22.2	*64.9*	*86.0*				
AJN	23.3	25.5								
EGrY	17.5	19.1	1.4	13.2	*8.0*	*69.1*				
OSN	20.2	22.1	8	18.2	*39.6*	*82.3*				
PFN	22.5	24.6	21.9	26.3	*97.3*	*106.8*				
HBS	26.3	28.8	10	24.2	*38.0*	*84.0*				
MHN	29.1	31.8	17.5	18.9	*60.1*	*59.4*				
means	***23.3***	***25.5***	**12.4**	**20.5**	***51.3***	***81.3***				
sd	***3.5***	***3.9***	***7.4***	***4.7***	***30.2***	***16.2***				
RMcE	30.9	33.8	15.2	26.9	*49.1*	*79.5*	18.4	12.6	*59.5*	*37.2*
CKN	17.9	19.6					8.0	15.6	*44.6*	*79.6*
JMK	19.3	21.1	11.7	5.4	*60.6*	*25.6*	27.9	6.2	*144.5*	*29.4*
EMcE	28.3	31.0	10.0	17.9	*35.3*	*57.8*	11.2	21.7	*39.6*	*70.1*
EGY	21.4	23.5	5.7	35.9	*26.6*	*153.0*	1.8	35.9	*8.4*	*153.0*
PFH	21.1	23.1	13.6	14.2	*64.3*	*61.4*				
DHD	24.2	26.5	19.4	21.7	*80.2*	*82.0*	16.1	30.6	*66.5*	*115.6*
SWS	24.1	26.3	11.7	20.5	*48.6*	*77.9*	19.6	3.8	*81.5*	*14.4*
PHS	24.2	26.5	7.7	20.4	*31.8*	*77.1*	24.0	17.5	*99.2*	*66.1*
means	***23.4***	***25.6***	**12.5**	**20.4**	***49.6***	***76.8***	**14.7**	**18.1**	***68.0***	***70.7***
sd	***4.2***	***4.5***	**4.3**	**8.9**	***18.2***	***36.0***	**8.6**	**11.1**	***41.4***	***46.1***
POE	22.7	24.8	10.3	16.3	*45.4*	*65.6*	18.8	24.4	*82.8*	*98.4*
GMN	17.6	19.3	10.6	14.3	*60.3*	*74.1*	18.8	12.2	*106.4*	*63.1*
LJS	24.4	26.6	20.6	22.1	*84.7*	*82.9*	7.7	0.4	*31.7*	*1.4*
JCN	21.9	24.0					15.9	26.7	*72.7*	*111.6*
EPY	22.8	25.0	13.1	6.3	*57.4*	*25.1*	13.2	13.0	*57.6*	*52.0*
JGH	20.2	22.1	7.8	21.4	*38.8*	*96.9*				
NHN	21.0	23.0								
means	***21.5***	***23.5***	**12.3**	**16.1**	***57.3***	***68.9***	**13.1**	**15.3**	***70.3***	***65.3***
sd	***2.2***	***2.4***	**4.9**	**6.4**	***17.6***	***27.1***	**4.6**	**10.6**	***27.9***	***43.3***

Table 4.8.3.9.3. **24 hr urinary inorgnic sulphate output (mmoles) before (24i) and after (24ii) oral load of Methionine or NAC and % recovery of dose administered.**

4.8.3.10. Micronutrient antioxidant status and supplementation

Serum Selenium

Table 4.8.3.10.1. reports the results obtained for the 24 subjects at baseline and in the patients after 5 and 10 weeks oral supplementation with *Bioantox* tabs. *i* qid. When these results are plotted, Figure 4.8.3.10.1. it can be seen that control subjects had values that were low compared to the laboratory's historical normal range, as did individuals with RAP. The mean value for the control group was higher than that observed in patients with CP, 85.3 vs. 61.3 μg/l ($p < 0.005$). Levels in subjects with a history of RAP did not differ from controls but were themselves higher than in the patients with CP ($p < 0.05$).

Five weeks oral supplementation resulted in elevation of serum selenium to normal or supra-normal levels, in all but one patient. For this young lady non-concordance was assumed but not admitted. By ten weeks, *Bioantox* tab *i* qid had elevated values in all patients above the lower limit of 'normal'. The rise in serum selenium over the base line measurements at both five and ten weeks, was highly significant for both the RAP and CP groups ($p < 0.01$). The observed increase in concentration appeared to be complete by 5 weeks therapy for no further significant rise occurs between 5 and 10 weeks, ($p = 0.31$).

Plasma Vitamin C

Table 4.8.3.10.2. reports the results obtained for plasma vitamin C in the 24 subjects at baseline and in the patients after 5 and 10 weeks oral supplementation with *Bioantox* tabs *i* qid. When these individual results are plotted, figure 4.8.3.10.2., it can be seen that control subjects had values that were spread across the laboratory's reference range. Individuals with RAP and CP had values that were predominantly in the lower tertile of the normal range, $p < 0.05$ for each versus the control group.

Oral supplementation resulted in elevation of the mean plasma vitamin C into the upper tertile in the two patient groups, Table 4.8.3.10.3. However, the lady in whom serum selenium did not rise, also failed to elevate vitamin C after

5 weeks supplementation, supporting non-concordance. Supplementation elevated plasma vitamin C to levels comparable with the control values by the end of 5 weeks therapy. This rise in plasma vitamin C was significant at the $p < 0.001$ level, when subjects with RAP and CP were combined into a single patient group. No difference was detected in plasma vitamin C concentration, between the two patient groups either before or after 5 and 10 weeks *Bioantox*.

	Controls		**Recurrent acute pancreatitis**		**Chronic pancreatitis**		**All pancreatitic patients**	
	mean	*s.d.*	*mean*	*s.d.*	*mean*	*s.d.*	*mean*	*s.d.*
Baseline	13.8	7.0	7.7	4.1	6.1	2.3	6.5	2.7
5 weeks			16.7	2.6	16.9	4.6	16.8	3.4
10 weeks			14.7	4.1	17.0	3.5	15.6	3.9

Table 4.8.3.10.3. Plasma vitamin C μg/ml prior to and after *Bioantox* supplementation, where ☐ represents a deficit in concentration at the $p < 0.05$ level with respect control baseline values, and ▒ an increase over the all-pancreatitic patient group prior to supplementation at the $p < 0.001$ level.

Serum fat soluble vitamins

Serum β-carotene, α-tocopherol and retinol were analysed in three or more s patient from each group, prior to and after 10 weeks oral supplementation. These results are depicted in Figures 4.8.3.10.3. - 5 respectively.

β-carotene concentration rose with supplementation in both patient groups. Due to the small numbers of patients analysed this rise did not reach statistical significance in either patient group, but did so when the groups were combined, $p = 0.02$.

Patients with RAP were found to have higher baseline α-tocopherol levels than those with chronic pancreatitis p = 0.03. Levels in both groups rose after taking *Bioantox* for 10 weeks with the mean of the combined group of patients with pancreatic disease increasing from 12.8 +/- 3.4 to 22.6 +/- 8.5 μg/ml (mean +/- 1 s.d.), p = 0.01.

The results for mean serum retinol demonstrated a difference at the time of entry into the study between fasting levels in subjects with RAP, 0.72 +/- 0.1, and CP 0.47 +/- 0.13 mg/l, $p < 0.05$. Following oral supplementation a rise in serum retinol concentrations was not confirmed.

4.8.3.11. Markers of free radical activity.

9,11 / 9,12 Linoleic Acid (% Molar Ratio)

A limited number of baseline samples were analysed for 9,11-linoleic acid and 9,12-linoleic acid from all three challenge groups, Table 4.8.3.11.1. No difference was detected in the molar ratio of any of the groups compared to one another or to historical laboratory controls. Figure 4.8.3.11.1. demonstrates the mean (+ 1 s.d.) values for the molar ratios of 9,11 / 9,12 linoleic acid in the study groups. As no difference in levels was detected before supplementation follow-up specimens were not analysed.

Summary of micronutrient antioxidant status and marker of free radical activity in challenge studies.

- At the time of recruitment serum selenium is lower in CP than RAP or controls
- Plasma vitamin C is lower in patients with pancreatitis than controls.
- In patients with pancreatic disease 5 weeks AOT elevates serum selenium and plasma vitamin C to within or above the normal range.
- AOT resulted in an increase in serum β-carotene and α-tocopherol levels.
- 9,11 / 9,12 linoleic acid molar ratios did not differ between groups

	Serum selenium µg/l		
Controls	**Time 0**	**5 weeks**	**10 weeks**
HBS			
PFN	82		
EGrY	80		
MHN			
AJN	87		
GKY	96		
NST	94		
OSN	73		
mean	*85.33*		
RAP Patients			
RMcE	77	147	
EGY	92	175	192
DHD	64	173	184
PHS	85	183	202
CKN	102	121	
EMcE	72	113	91
JMK	76		165
SWS	61	143	151
PFH	78		
mean	*78.56*	*150.71*	*164.17*
CP Patients			
JCN	56	161	159
JGH	54	191	216
NHN	66		148
LJS	78	80 *	177
GMN	32	163	150
POE	79	207	217
EPY	64	151	139
mean	*61.29*	*158.83*	*172.29*

Table 4.8.3.10.1. Serum selenium concentration (µg/l) in challenge subjects before and during oral supplementation.

* indicates likely non-compliance to this time point.

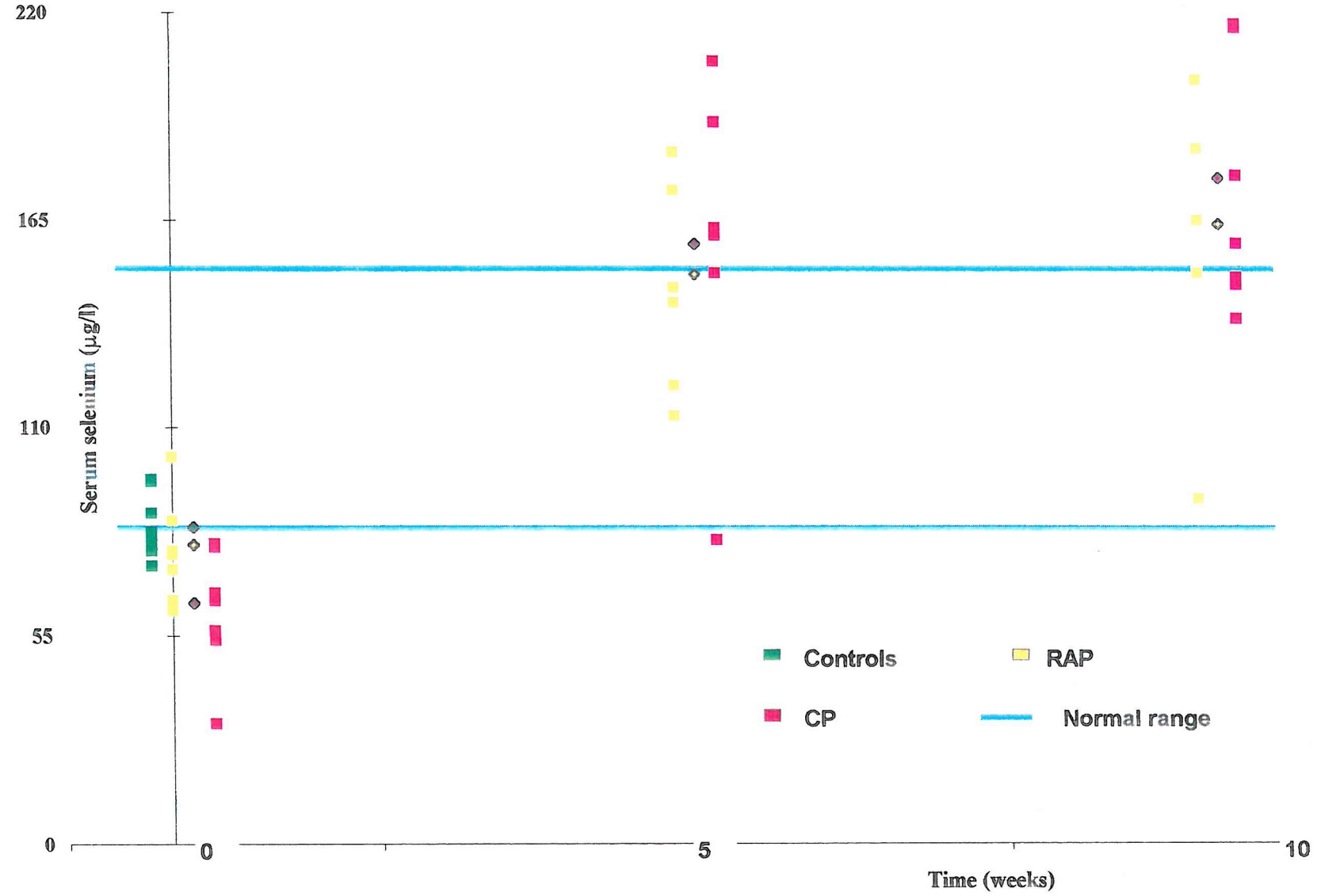


Figure 4.8.3.10.1. Individual serum selenium concentrations before and after oral supplementation

	Plasma vitamin C μg/ml		
Controls	**Time 0**	**5 weeks**	**10 weeks**
HBS	3.8		
PFN	21.7		
EGrY	14.4		
MHN	24.3		
AJN	11.7		
GKY	16.2		
NST	12.1		
OSN	6.3		
mean	*13.8*		
RAP Patients			
RMcE	4.4	15.8	8.4
EGY	7.7	14.5	18.3
DHD	10.4	19.2	15.8
PHS	5.1	20.5	19.4
CKN	5.1	13.9	12.3
EMcE	4.6	13.8	9.7
JMK	12.6	19.1	18.0
SWS	4.3	16.9	15.3
PFH	15.5		
mean	*7.7*	*16.7*	*14.6*
CP Patients			
JCN	5.8	23.9	13.5
JGH	4.7	15.5	16.2
NHN	7.2		19.4
LJS	7.9	9.8 *	18.2
GMN	2.6	16.6	13.4
POE	5.2	16.7	17.6
EPY	9.6	18.7	22.8
mean	*6.1*	*16.9*	*17.3*

Table 4.8.3.10.2. Plasma Vitamin C concentration (μg/ml) in challenge subjects before and during oral supplementation.

* indicates likely non-compliance to this time point.

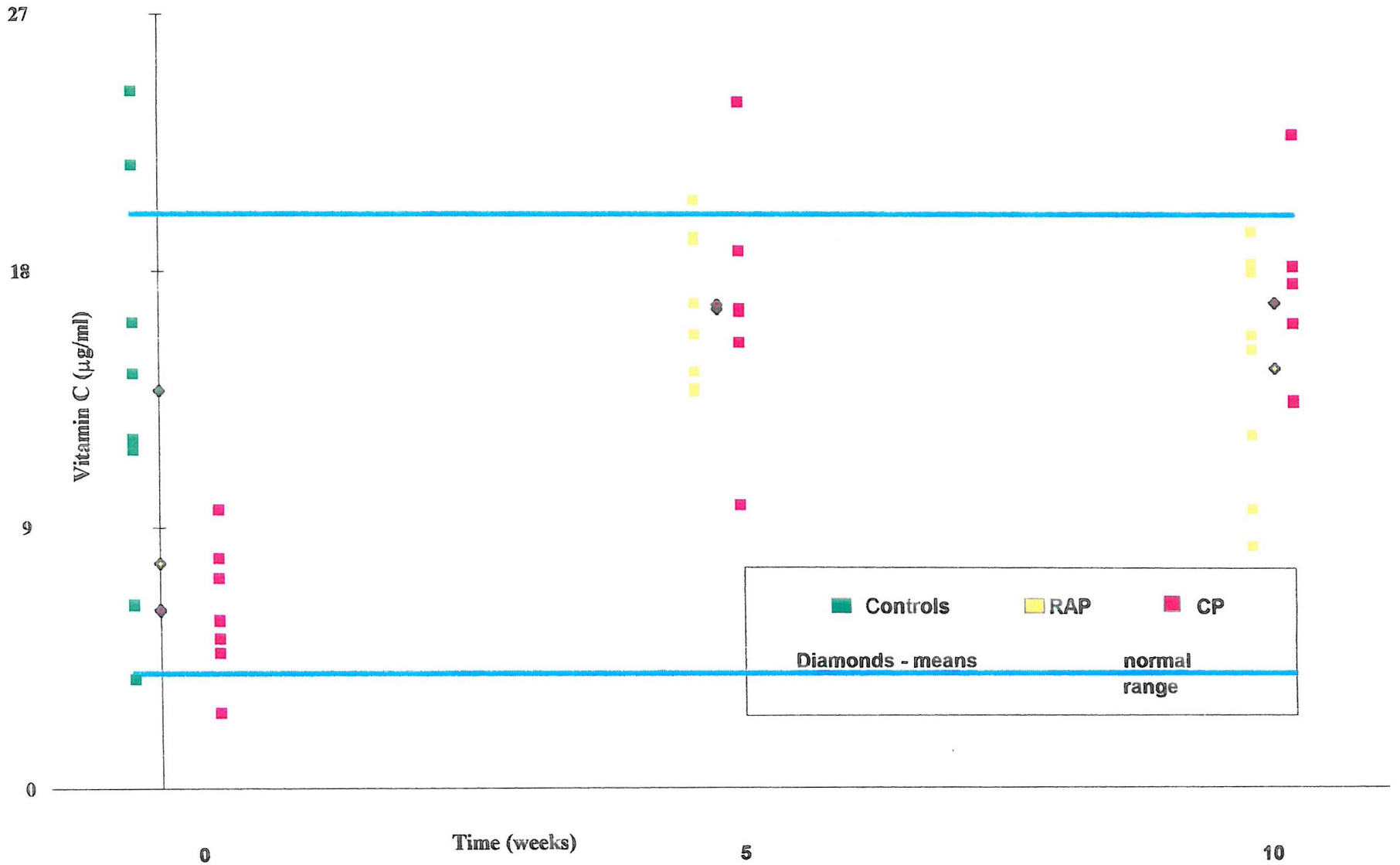


Figure 4.8.3.10.2. Individual plasma vitamin C (µg/ml) before and after oral supplementation

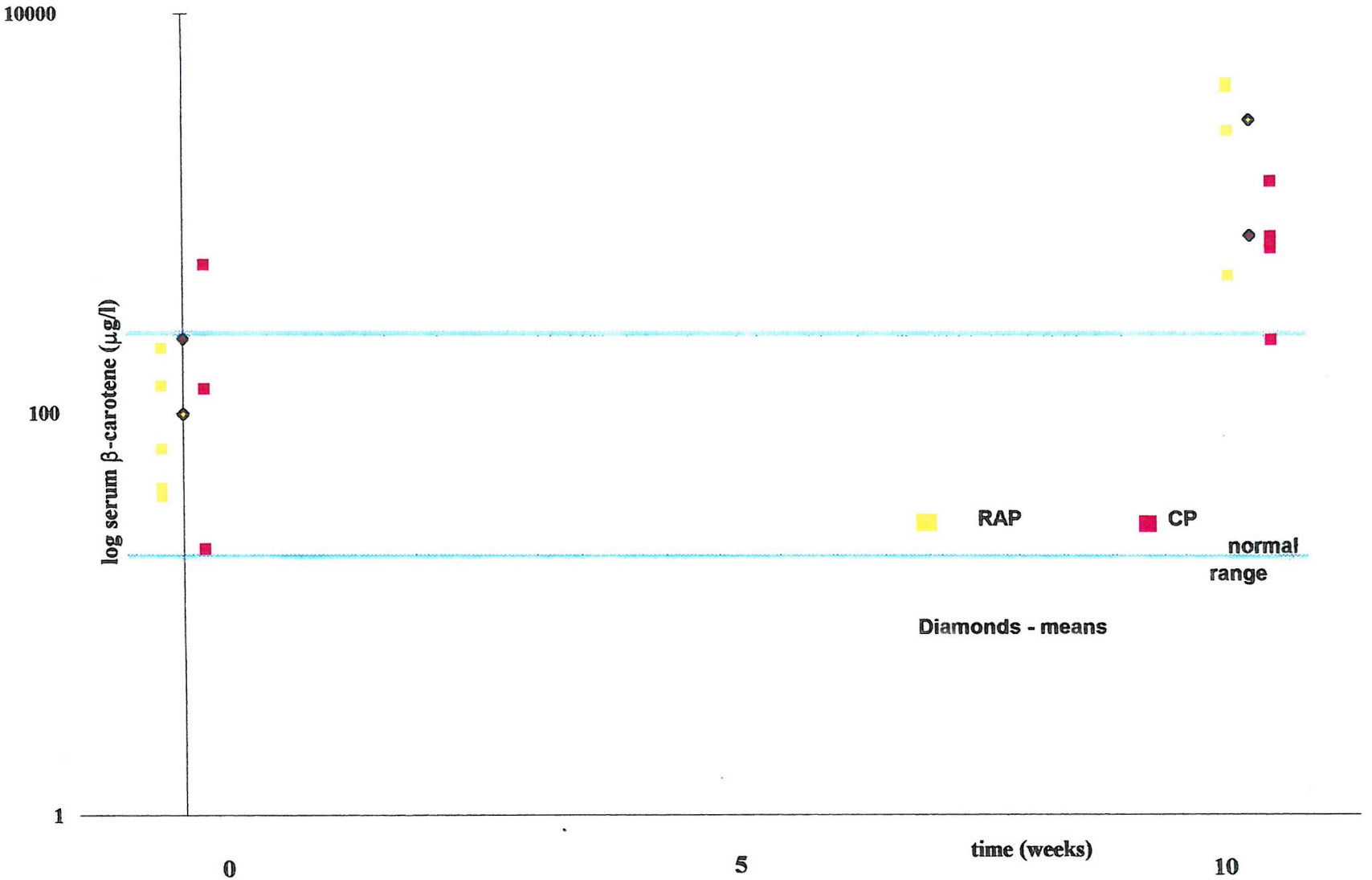


Figure 4.8.3.10.3. Individual serum β-carotene concentrations before and after oral supplementation (logarithmic scale)

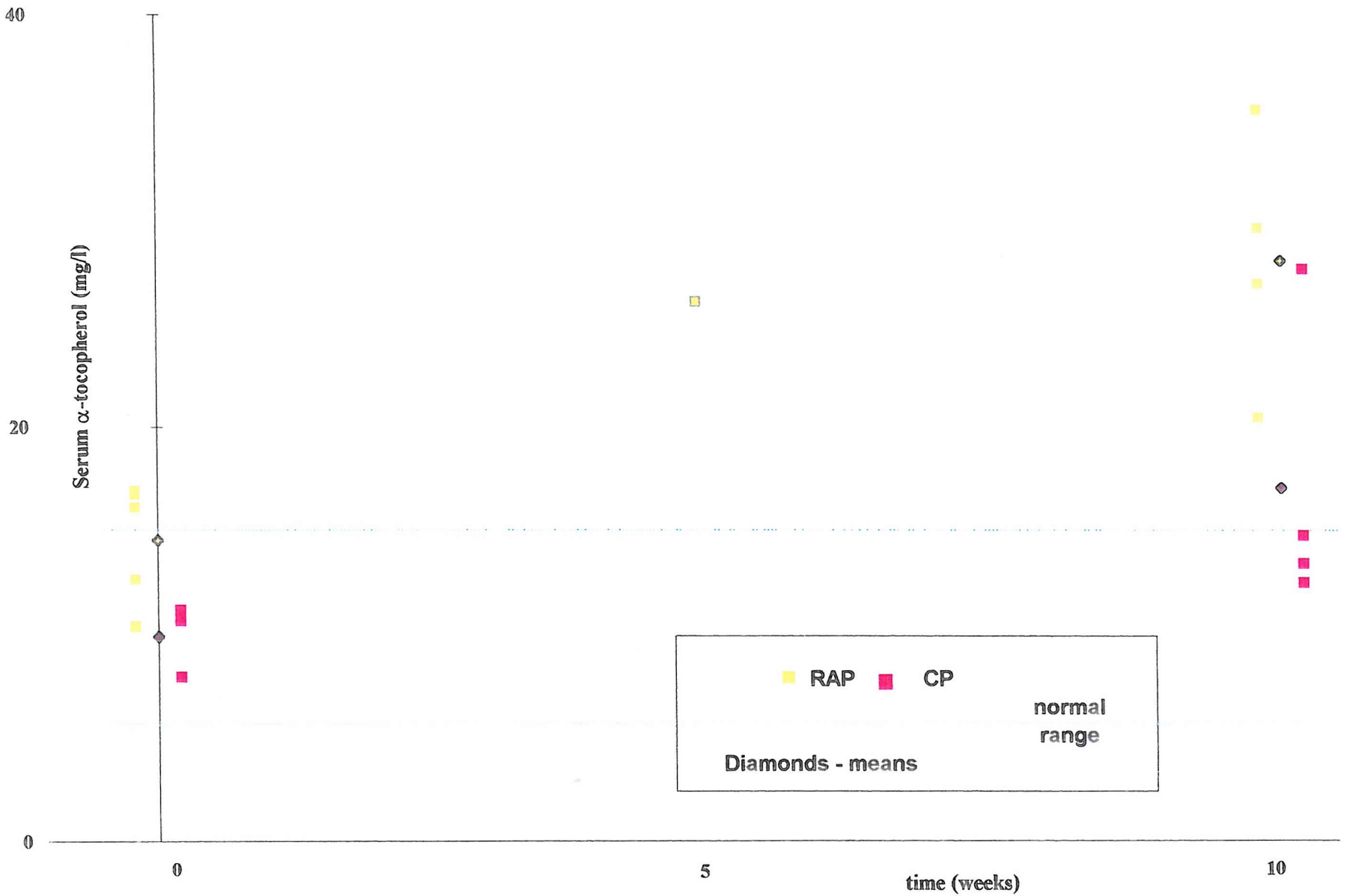


Figure 4.8.3.10.4. Individual serum α-tocopherol concentrations before and after oral supplementation

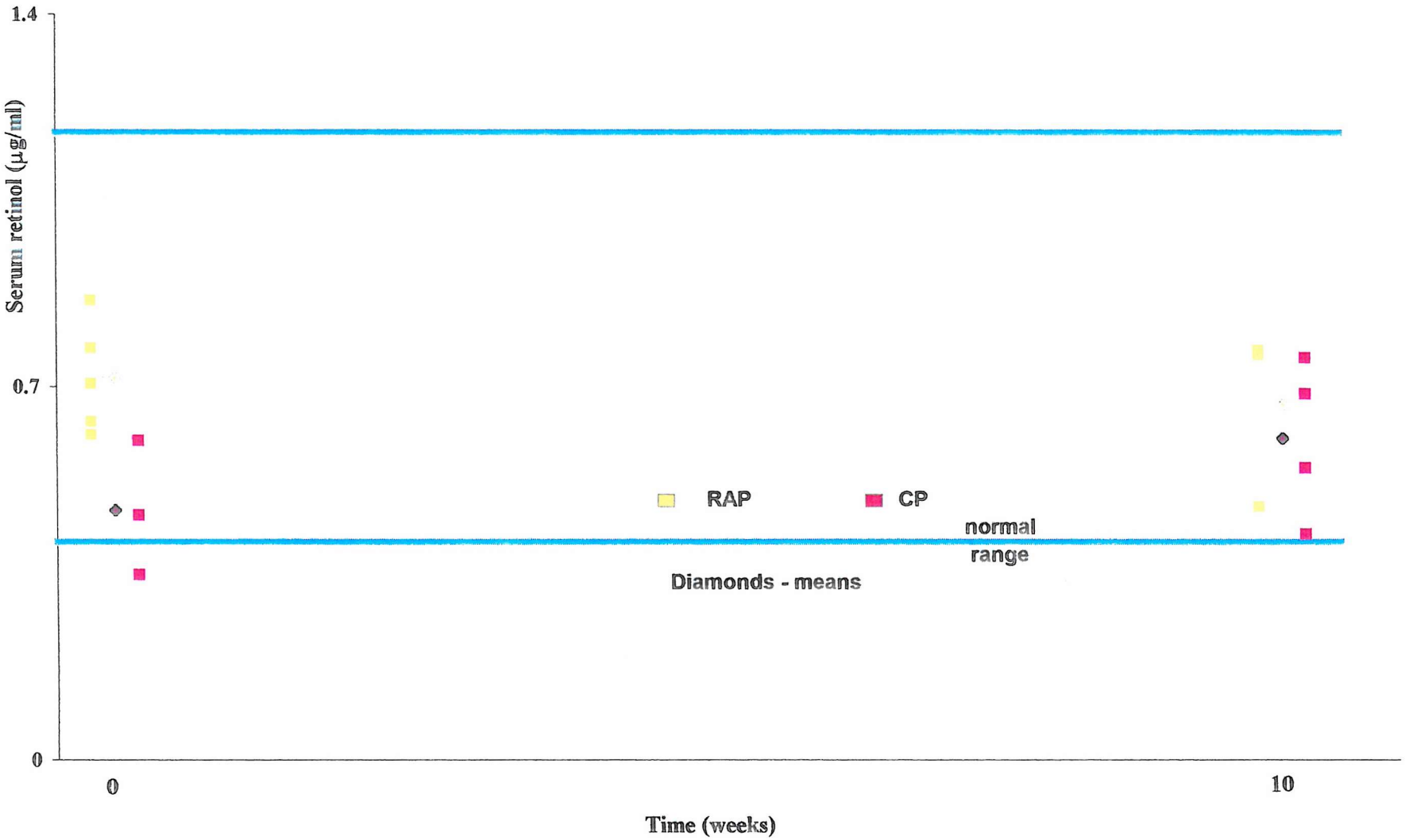
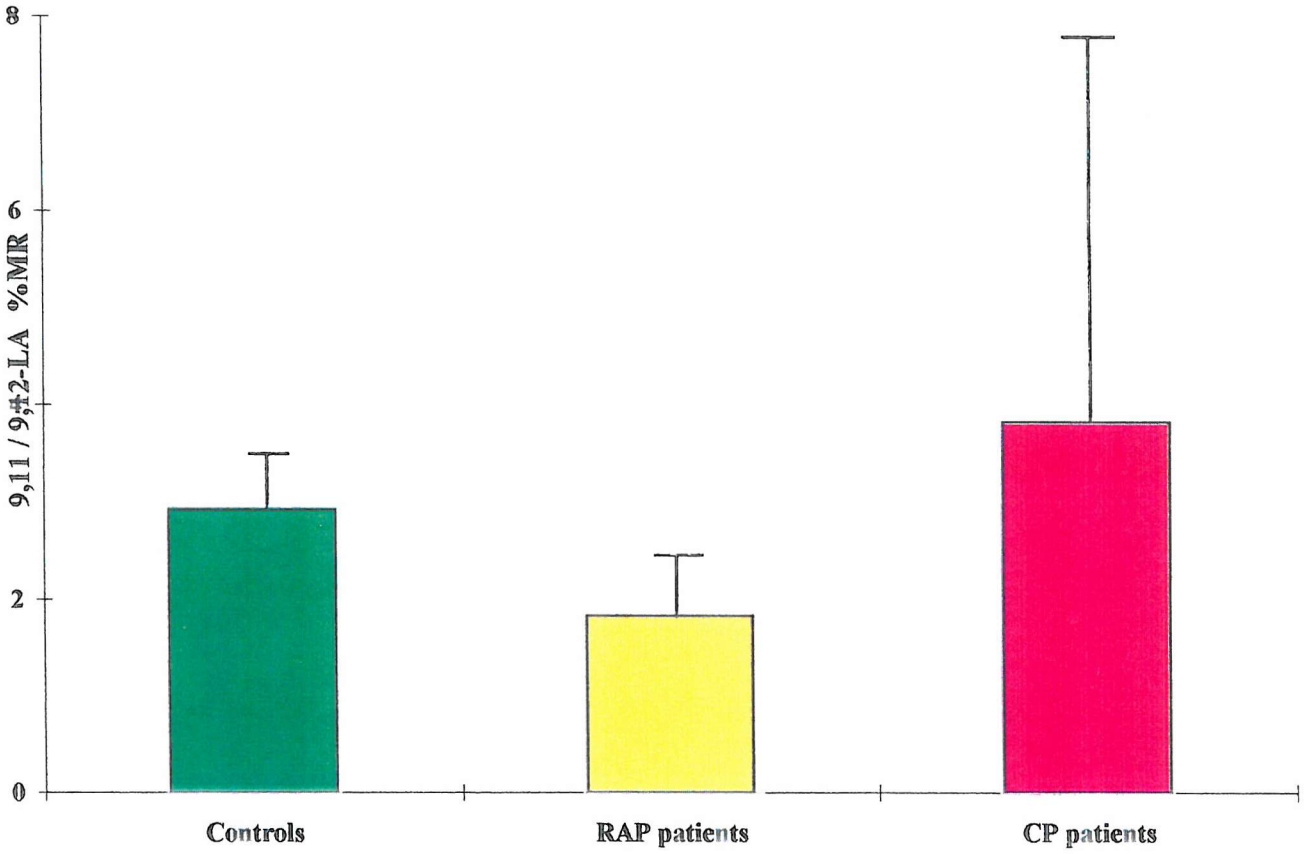


Figure 4.8.3.10.5. Individual serum retinol concentrations before and after oral supplementation



9,11 / 9,12 L.A. % MR					
Controls		RAP patients		CP patients	
PFN	3.19	EGY	2.08	JCN	3.61
AJN	2.45	SWS	1.31	JGH	1.51
GKY	3.39	PFH	2.08	NHN	1.55
NST	2.67			LJS	1.74
				POE	10.75
mean	2.93		1.82		3.83
sd	0.44		0.44		3.96

Figure 4.8.3.11.1. 9,11 / 9,12 Linoleic Acid Molar Ratio percentages (Group Mean, 1 s.d. and values)

		tau	ser	gly	val	1/2-cys	meth
Armstrong 1973							
men	*mean*	162.0	114.0	236.0	252.0	118.0	32.0
	sd	60.0	23.0	42.0	37.0	17.0	6.0
women	*mean*	141.0	127.0	300.0	209.0	109.0	27.0
	sd	57.0	29.0	114.0	31.0	24.0	5.0
Chawla 1984						**1/2-cys**	
controls	*mean*	69.4	?	273.0	206.6	79.4	31.9
	sd	8.3	7.6	20.1	6.6	7.6	2.6
cirrhotics	*mean*	47.9	102.2	312.9	101.0	88.6	59.0
	sd	4.3	25.8	62.1	14.4	22.4	20.6
Roth 1985						**$(cys)_2$**	
controls	*mean*	51.3	102.2	207.8	213	96.1	22.1
	sd	31.2	26.7	51.6	42	26.4	4.5
AP	*mean*	68.7	68.8	154.8	161.6	85.4	17.8
	sd	83.1	26.8	94.4	52	35.1	7.5
Martensson 1986						**1/2-cys**	
controls	*mean*	72				90	22
	sd	6				6	3
Bottiglieri 1987							
controls	*mean*						28
	sd						3
Current challenge studies						**$(cys)_2$**	
controls	*mean*	76.6	112.5	246.6	242.1	116.4	23.8
	sd	17.7	21.3	60.4	51.9	20.6	4.4
RAP	*mean*	99.6	103.1	240.2	252.2	125.7	22.7
	sd	28.7	28.1	89.3	44.9	20.6	3.3
CP	*mean*	82.4	93.2	235.8	183.6	118.6	20.8
	sd	26.1	22.3	61.6	35.8	24.9	4.8

Table 4.8.3.12.1. **Reported plasma amino acid mean and s.d. concentrations (μmol/l).**

4.8.4. Summary of challenge study results

- Fasting plasma methionine, cystine, taurine, glycine and valine and wbGSH levels were normal in healthy controls and patients with pancreatic disease.
- Oral methionine loading results in a detectable plasma peak between 90 and 180 minutes.
- Plasma methionine falls progressively for the first three hours after oral NAC, before returning to pre-dose level over the next three hour period.
- 10 weeks AOT (containing 1.6g methionine daily) does not alter fasting methionine concentrations or the peak value observed following an oral load.
- There was a trend for the plasma methionine levels in patients with CP to be lower than those of controls, thirty minutes after a methionine load, irrespective of antioxidant status. No differences at other times were observed.
- Plasma methionine half-life following an oral load of methionine, were similar in healthy subjects and individuals with a history of recurrent acute and chronic pancreatitis.
- AOT had no significant effect on the t½, V_d and MCR of plasma methionine following an oral methionine load.
- AOT does not alter the mean AUC of plasma methionine, after an oral challenge of methionine or NAC, in patients with a diagnosis of pancreatitis.
- Oral NAC results in elevation of plasma cystine, with a peak between 30 and 90 minutes. The cystine t½ and AUC of cystine profiles of healthy individuals and patients with quiescent pancreatitis, did not differ before or after AOT.
- Oral loading with NAC or methionine has no effect on plasma cystine, taurine, glycine, serine or GSH and wbGSH profiles, or the AUC of the controls or patients, irrespective of antioxidant status.
- Plasma serine concentration alters after oral NAC, but at different time points and in opposite directions in different subject groups and it is thus doubtful whether these reflect real changes.
- A fall in plasma valine level was generally observed 270 minutes after oral loading with NAC or methionine, irrespective of the group studied.

- Fasting mean plasma valine concentration in CP were lower than in patients with RAP. These differences persisted throughout the 9 hour profile and are reflected in differences in AUC in the methionine challenge pre-AOT.
- 10 weeks AOT appeared to have no effect on plasma valine or wbGSH profiles.
- Plasma GSH appears to be lower in RAP and CP than controls subjects, before AOT.
- AOT does not elevate plasma GSH of patients into the normal range.
- $UiSO_4$ output from patients with quiescent CP is low, but not significantly lower than healthy individuals or subjects with RAP.
- Following an oral load of NAC $UiSO_4$ output peaks by 6 hours. After oral methionine the peak $UiSO_4$ output is more delayed.
- Over 50% of the sulphur content of an oral load of NAC or methionine, is excreted as $UiSO_4$.
- At the time of recruitment serum selenium is lower in CP than RAP or healthy controls.
- Plasma vitamin C is lower in patients with pancreatitis than controls.
- In patients with pancreatic disease, 5 weeks AOT elevates serum selenium and plasma vitamin C to within or above the normal range.
- AOT results in an increase in serum β-carotene and α-tocopherol levels.
- 9,11 / 9,12 linoleic acid molar ratios did not differ between groups.

4.9.0. Discussion

Methionine transsulphuration: recapitulation of premises

The hypothesis that methionine metabolism is inherently disturbed in pancreatitis is not unequivocally supported by the studies described in this chapter. However, arguments exist for and against the premise.

4.9.1. Evidence against the premise of transsulphuration disturbance in pancreatitis.

We were unable to confirm reports of abnormal methionine metabolism in patients with pancreatitis. The first of these previous reports found elevated leukocyte methionine in chronic relapsing pancreatitis (CRP) (Mårtensson 1986a), while subsequently, whole-blood free methionine concentrations were higher in acute pancreatitis than controls, (Viña 2001). We found no difference between the fasting baseline plasma methionine levels of healthy controls and the two groups of individuals with quiescent pancreatitis (RAP and CP). In the two papers cited above, the study subjects had evidence of active pancreatic inflammation at the time of analysis, as demonstrated by elevated levels of serum amylase and acute symptomatology. All but one of our subjects had inactive disease and were either 6 weeks or more post an attack of acute pancreatitis or had evidence of quiescent chronic pancreatitis, without active inflammation.

The one patient in the current study who did have evidence of active acute-on-chronic pancreatitis (POE), had a larger AUC (Graph 4.8.3.1.3) for the plasma methionine concentration profile following an oral load, than the remaining 6 patients in the chronic pancreatitis group (prior to AOT), 2587 versus a mean of 1575 μmol/l/hr for the remaining patients with CP, n = 6. During the 10 weeks AOT, this patients' alcoholic hepatitis and acute pancreatic inflammation settled as evidenced by normalisation of hepatic plasma transaminases and serum amylase

levels. This clinical improvement was associated with a fall in the total AUC for plasma methionine concentration to 1913 μmol/l/hr while the average for the remaining patients was unchanged at 1347 μmol/l/hr. In neither this man, nor any of the other subjects studied, was fasting plasma methionine altered by 10 weeks AOT containing 1.6g L-methionine daily. The average half-life of methionine in the subjects studied was approximately 4½ hours. Bioantox was prescribed six hourly and thus over 10 weeks a rise in methionine would have been expected. However, for each challenge study, they were asked to omit supplements from the previous evening and thus over 2 half lives would have elapsed by the time the baseline sample was obtained and there would be little residual methionine at that point.

In Mårtensson 1986a, all six patients studied had alcohol-related CRP and also had disturbed liver function tests. Disturbances of methionine transsulphuration are well recognised in both cirrhosis (Horowitz 1981, Marchesini 1992, Bianchi 2000) and alcoholism (Guerri 1980, Bleich 2000a). Thus the changes in leukocyte methionine and urinary sulphate might have been a consequence of alcohol-related liver disease rather than acute pancreatitis. No such hepatic inflammation was present in the current study's subjects.

Secondly, the half life of plasma methionine (t½) following oral loading was the same in control subjects and patients with diagnoses of recurrent acute or chronic pancreatitis, Table 4.8.3.1.5. & Figure 4.8.3.1.21. The methionine concentration versus time curves for the three subject groups following oral loading with methionine before AOT, are shown in Figure 4.8.3.1.6 and after AOT, in Figure 4.8.3.1.7. An apparent difference between the peak value at 180 minutes after an oral methionine load in the CP group of 277.6 +/- 101.7 μmol/l before AOT, and 216.8 +/- 69.9 afterwards (mean +/- 1s.d.) is not significant, Students t-test: $p = 0.08$. No differences were present between any other groups at any time point. Again, these results differ from studies performed over 50 years ago in California, in which patients with cirrhosis or hepatitis were shown to have a reduced clearance

rate of plasma methionine compared to controls (Harper 1947, Kinsell 1947). In those studies, humans with liver disease were loaded with an intravenous injection of 1.5g DL-methionine to quantify methionine clearance rates (Harper 1947). They found fasting plasma methionine concentrations to be between 0.46 - 1.48 mg/cl (310 - 990 μmol/l) and following an intravenous load of 50ml of 3% DL-methionine the plasma clearance to be 0.41 mg/cl/hour (270 μmoles/l/hr). These figures obtained by microbiological assay after intravenous administration, compare to our HPLC methodology following oral loading with an average fasting plasma concentration = 21μmol/l and a plasma half life (t½) = 254 - 272 minutes.

More recently, Horowitz *et al* reported a plasma methionine t½ of 146 +/- 10 (SEM) minutes in their control group, and 458 +/- 81 minutes in experimental subjects (biopsy-proven cirrhotics) after an oral load with 50 mg/kg L-methionine. In this study the 9 controls had an age range 23 - 32 (mean not given) compared to a patient mean age of 52 years (Horowitz 1981). In our study, the average age of >40 years did not differ between the control and patient groups and the t½ for controls of 271 +/- 40 minutes lay between the values for controls and subjects reported by Horowitz. Whether these age differences fully account for this discrepancy is unlikely.

In a very recent report, subjects were challenged with oral methionine (100mg/kg) and a methionine t½ of 292 +/- 69 minutes was found in the control group (age range 31 - 68 years, median omitted). As in the Horowitz *et al.* study, cirrhotics had prolonged values, 720 +/- 427 minutes (age range 45 - 68 years, median 55), (Bianchi 2000). These results suggest that cirrhosis / chronic liver disease rather than age is the main factor responsible for prolonging methionine half-life. It is thus not surprising that as the subjects in our study do not have significant liver disease, that their t½ is similar to the healthy controls.

Returning to the concept that methionine half-life might lengthen with age, it has been suggested that the availability of the transsulphuration cofactors (vitamin B_{12}, folic acid and pyridoxal 5-phosphate) fall with age (Exton-Smith 1980). Even when blood values are within the normal range, intracellular levels may not be (Meenan 1996). Their availability influences homocysteine levels (Cravo 1996), and supplementation, even in the presence of normal blood concentrations can reduce homocysteine levels (Naurath 1995). Could availability of cofactors explain a tendency for the half-life of methionine to increase with age ?

Thirdly, our expectation that oral methionine loading would have no detectable effect on plasma cystine levels, was confirmed. Loading with NAC generates cysteine (Dringer 1999), which feeds into the transsulphuration pathway below the rate limiting cystathionine synthase stage, Figure 1.3. However, no difference in the cystine t½ following this oral load of NAC was observed between the groups examined. Thus cystine t½ was 480.2 +/- 71 minutes for controls (mean +/- SEM), 343 +/- 31 for patients with CP before AOT and 307 +/- 112 for the pancreatitis patients as a combined group post AOT, Table 4.8.3.2.5. This half life is of the order of 7 times slower than that reported for cysteine following oral NAC at a dose of 30mg/kg (Burgunder 1989).

4.9.2. Evidence supporting the premise of transsulphuration disturbance in pancreatitis

I now turn to evidence that might support a disruption of the transsulphuration pathway in the subjects studied. Despite normal fasting plasma methionine concentrations, low plasma GSH was observed in the CP group. This is not entirely unexpected or irrational. GSH is an end-product of transsulphuration as are taurine and sulphate. If methionine levels are maintained yet plasma GSH is low, then anomalies of transsulphuration intermediates might be expected. SAMe, the first intermediate of transsulphuration is synthesized by, methionine adenosyltransferase

60 and 2000mg/kg in 24 hours in ameliorating cerulein-induce experimental pancreatitis.

The relationship between intracellular and plasma GSH is poorly defined. However, plasma GSH concentrations have traditionally been used to evaluate inter-organ metabolism. But we did not document any difference in intracellular levels (whole blood GSH) but did see lower plasma levels in the patient groups. The significance of this is unclear, for as 98% of total blood GSH is intracellular, some have suggested plasma levels to be unimportant. Erythrocytes express the enzymes required to synthesise large quantities of GSH and have been shown to carry the majority of GSH between organs (Dass 1992). Whether depressed plasma GSH levels represent increased organ uptake, decreased release from intracellular sites, accelerated uptake by intracellular sites of synthesis attempting to maintain an adequate supply or a combination is unknown. Whatever the explanation, methionine supplementation does not seem to elevate plasma GSH or to that matter whole blood GSH.

Taurine, traditionally considered an end product of the transsulphuration pathway, was found to be normal in fasting plasma of patients with pancreatic disease. Taurine is a potent antioxidant in its own right. It is recognised to be protective against ethanol-induced hepatic steatosis and lipid peroxidation (Kerai 1998), thioacetamide-induced hepatic cirrhosis (Balkan 2001) and streptozotocin-induce diabetes in rats (Chang 2000). A recent publication has reported that taurine supplementation is also able to protect rats from cerulein-induced pancreatitis (Ahn 2001). Given these observations one might have expected taurine to have been higher in subjects with a history of pancreatitis than the healthy controls. That it was not may imply a block or disturbance of transsulphuration.

4.9.3. Methionine loading studies

Interpreting the metabolic consequences of methionine loading requires an appreciation of the dynamic relation between reduced, oxidised and protein-bound homocysteine and other thiols in plasma (Mansoor 1992).

Loading humans with methionine was first reported by Harper et al 1947, who looked at methionine clearance in liver disease, as described above. Brenton *et al.* 1965, utilised oral L-methionine loading in the parents of three children affected with homocystinuria, at a dose of 0.1g/kg body weight, with the intention of demonstrating impaired cystathionine synthetase activity. In the same year the direct relationship between sulphur-containing amino acid intake and urinary inorganic sulphate was clarified (Sabry 1965), with methionine oxidation being held primarily responsible (Mudd 1972). These experiments are supported by studies on fasting healthy subjects again demonstrating that urinary inorganic sulphate is derived mainly from dietary amino acids (Mårtensson 1982). Dietary sulphur amino acid intake is important in inflammatory diseases such as pancreatitis, for an inadequate pre-morbid diet prevent the restoration of GSH concentrations that usually occurs in conjuction with a systemic inflammatory process (Hunter 1997).

The low plasma GSH in our patients with CP may well be a direct reflection of inadequate sulphur amino acid intake as demonstrated by the low urinary inorganic sulphate excretion level in this study and confirms previous dietary studies (Rose 1986). However, chronic relapsing pancreatitis is reported to reduce the amount of sulphur amino acids oxidised to inorganic sulphate (Mårtensson 1986a). Hence dietary insufficiency may be only part of the answer. Low blood GSH often reflects reduced hepatic synthesis / release (Anderson 1980) and the rate of its synthesis is governed at least to a degree, by the rate of cysteine precursor uptake by hepatocytes (Banks 1994). Human pancreatic levels have been reported as being low in patients coming to pancreatectomy for painful chronic pancreatitis, or severe acute haemorrhagic disease, compared to controls drawn from organ donors (Schoenberg 1995).

Methionine loading has been used to identify individuals with genetically impaired homocysteine metabolism. It is important to use L-methionine rather than DL-methionine for D-methionine is poorly utilised by adult humans (Stegink 1986). L-methionine loading was utilised to examine the hypermethioninaemia associated with cirrhosis, by Horowitz in 1981. His group used a dose of 50mg/kg ideal body weight to demonstrate a block in transsulphuration 'above homocysteine synthesis' in their patients. This protocol was the basis of the current study in pancreatitis patients. Work from Malmö, Sweden needs noting, for it examined whether a methionine-rich diet altered methionine clearance or homocysteine levels. It concluded that the daily methionine intake does not affect the methionine loading test (Andersson 1990). This is important in our work, for we examined pancreatitis patients known to have low methionine intakes (Rose 1986) before and after supplementation with 1.6g methionine daily for 10 weeks. The same Swedish group looked at the effect of gender and aging on pre- and post- loading homocysteine levels. They concluded that men (especially older men), have higher homocysteine levels than age matched women and that these findings reflected decreasing transsulphuration pathway cofactor concentrations e.g. pyridoxine, folic acid and vitamin B_{12} (Andersson 1992). Similarly, plasma homocysteine levels are unaffected by increasing dietary methionine intake for a week as long as folate and the relevant B-vitamin cofactors are not limited (Ward 2000). Blom and coworkers found that premenopausal women had lower homocysteine levels in both the fasting state and after methionine loading (Blom 1988). Samples from the current study remain stored for future homocysteine analysis.

The studies comprising this chapter were amongst the first to examine the effect of methionine loading on blood glutathione levels. A recent report from Italy, found an increase in plasma GSH in controls after methionine challenging. Interestingly, this response was impaired / absent in their patients, a group of cirrhotics (Bianchi 2000). In comparison, our results revealed no increase to the plasma GSH levels in controls nor any change in individuals with a history of pancreatitis following either an oral methionine or NAC load. The report from Bologna, Italy also found that

methionine loading caused a rise in cysteine in controls but not the cirrhotic group, although no difference in the cystine time course was found between groups. Furthermore, although plasma taurine levels increased three hours after oral methionine, the time course did not differ from patients with liver disease.

However, the pioneering work of Davies 1986, found that whilst both methionine and NAC if present at the time of contact or soon afterwards, could protect against paracetamol-induced injury of isolated hepatocytes, it was only NAC that bolstered GSH when given later. These observations are in keeping with earlier reports, that glutathione synthesis is more sensitive to cysteine than methionine supply (Tateishi 1981, Reed 1977) and that NAC but not methionine or L-2-oxothiazolidine-4-carboxylate (OTC) serve as cysteine donors for GSH synthesis in cultured neurons (Dringen 1999). Furthermore, OTC attenuated the fall in GSH and the severity of cerulein-induced pancreatitis in mice (Lüthen 1997).

The time course of the metabolic consequences of methionine loading has also been the focus of work. The standard methionine loading test quantitates homocysteine concentration four hours after ingestion. At least one group advocates reducing this period to two hours (Bostom 1995). This would not have been appropriate in the studies described in this chapter as plasma methionine peaked between 90 and 180 minutes after loading. The time difference is likely to be due to the form of methionine administered. We gave tablets, whereas the Horwitz and Bostom groups gave a solution, which will accelerate absorption. It may simply be this factor that resulted in urinary inorganic sulphate being increased by NAC by 30 minutes (given as a solution) compared to methionine after 60 - 90 minutes (given as tablets). That methionine augments the top of the pathway, whereas NAC feeds via cysteine into the lower part may be another aspect.

Methionine loading can be utilised in assessing mild hyperhomocysteinaemia, a recognised risk factor for vascular disease (Clarke 1991), stroke (Perry 1995) and coronary heart disease (Nygård 1997). However, some authorities considered the

test superfluous, at least as far as being a screening tool for cardiovascular risk is concerned (Smulders 2000).

4.9.4. Amino acids in pancreatic disease

These studies have identified a number of differences in plasma amino acid concentrations between study groups. Due to the large number of samples involved, these were collected and analysed over an extended period. Previously, the stability of samples stored at minus 20°C has been shown to be at least 21 days (Bottiglieri 1987). We found that plasma amino acids of particular interest in this study, with the exception of cystine, remained stable for up to 5 years, when stored at minus 70°C.

We found that patients with CP had a lower mean fasting plasma valine 186.5 +/- 28.5 μmol/l than controls 246.1 +/- 51.5 μmol/l or subjects with RAP 252.2 +/- 38.4 μmol/l, Table 4.8.3.4.1. A similar pattern was found between a group of controls and patients with acute pancreatitis by Roth 1985, with patients also having lower valine. However, plasma branched-chain amino acid concentrations (leucine , isoleucine and valine) have been found to be increased not decreased in 'pancreatic diabetes' due to chronic pancreatitis, compared to primary diabetes or controls (Nakamura 1994). This group also reported an inverse correlation between plasma amino acid levels and glucagon, suggesting an important influence of this hormone on the balance between gluconeogenesis and glycogenolysis in determining plasma amino acid levels.

4.9.5. Oxidative stress

The pancreatitis patients in this study had low levels of selenium, vitamin C and glutathione, supporting previous observations (Braganza 1988a, Schofield 1991,

Uden 1992, Scott 1994). Free radical scavenging capacity would be impaired under such conditions and correction of this anomaly, would be expected to reduced the drive to inflammation, though the release of the 'pancreastasis' that ensues under oxidative stress. This is the rationale behind the oral micronutrient antioxidant supplement regime used to treat RAP and CP (Uden 1990a, Bilton 1994, Sharer 1995, Dite 1998). A similar mechanism pertains to the use of antioxidants in liver disease (Lieber 1997).

We found that *Bioantox* 4 tablets daily, corrected the subnormal serum selenium and plasma vitamin C by the end of 5 weeks. Although not an end point of this study, limited evidence for the efficacy of this therapy was obtained from the resolution of pain, and normalisation of hyperamylasaemia, during the period of supplementation in one patient (POE) whose symptoms at the time of recruitment were attributed to ongoing pancreatic inflammation. Furthermore, no patient with a history of RAP suffered a relapse during the study period and none of the subjects with chronic pancreatitis reported worsening of pain or had objective evidence of new acute-on-chronic disease. Similar findings were reported by our group over a decade ago when a reduction in background pain and relapse rate was observed in a 20 week double-blind double-crossover trial of a similar antioxidant regime (Uden 1990a). The comparison of pancreatitis to liver disease extends to the therapeutic benefit of exogenous modulation of the transsulphuration pathway:

i) Choline supplementation ameliorates parenteral nutrition associated hepatic steatosis (Buchman 1995).

ii) Intra-peritoneal GSH lessens acute liver injury induced by carbon tetrachloride in mice (Arosio 1997).

iii) Oral methionine improves the histological changes in alcohol-induced liver damage in rats (Parlesak 1998) and,

iv) SAMe may delay liver transplantation in less advanced alcoholic cirrhosis (Mato 1999).

The list is not exhaustive but further strands of research suggest that administration of transsulphuration pathway products e.g. SAMe (Arias-Diaz 1996) and GSH (Pena 1999), reduce the pro-inflammatory cytokine drive to inflammation, which may prove to be one mechanism by which AOT acts.

Chapter 5
Sulphur Amino Acid Metabolism, Pancreatitis and Oxidative Stress: Revisited.

5.1. Hepatocyte meets pancreatic acinar cell

Whereas the central role of methionine metabolism in the maintenance of cellular integrity, and of the pancreatic acinar cell in particular, has been known for some time (Véghelyi 1950), the recognition of its vulnerability to oxidative stress and potential application of this feature to the initiation of pancreatitis has been a relatively recent development (Mårtensson 1986a, Rose 1986, Uden 1990, Braganza 1991, 1998).

This connection is exemplified in studies of hepatotoxicity from paracetamol (Davies 1986) and carbon tetrachloride (Poli 1993). There are crucial differences, however, between the hepatocyte and the pancreatic acinar cell, insofar as sulphydryl and methyl group homeostasis is concerned. Thus, the GSH quota within hepatocytes is now known to be several degrees of magnitude greater than the pancreas (Githens 1991, Bray 1993, Neuschwander-Tetri 1997). This difference is in line with the intact liver's pivotal role in the metabolism of ingested xenobiotics conveyed through the portal vein and as the major site of synthesis for release into the circulation (Dass 1992). Yet it is now documented that the acinar cell and thus the pancreas itself, cannot fulfill its primary role as producer of digestive enzymes without GSH and methyl groups (Stenson 1983, Capdevila 1997).

Recent work in rats shows that pancreatic exocrine function is usually preserved under conditions of mild oxidative stress and involves the oxidation of GSH to GSSG by ascorbate in the mitochondria (Mårtensson 1993). However, any increase in free radical production above this baseline level is not well tolerated and this is irrespective of whether it is precipitated by the exposure to a triglyceride-rich (corn oil) meal or a dose of alcohol (Neuschwander-Tetri 1997). It is thus easy to see, that the direct entry of inhaled xenobiotics via the pancreas glands' rich arterial supply would be potentially devastating, for in contrast to the refurbishment of GSH when

depleted by the removal of free oxygen intermediates, *de novo* intracellular synthesis is required when GSH is lost through irreversible binding to xenobiotic metabolites formed by cytochrome P450 activity or prostaglandin synthesis.

Indeed, recent studies have reinforced work from Manchester (Braganza 1986a, McNamee 1994, Foster 1993, Sandilands 1990, Braganza 1987, 1993b, 1998) in identifying occupational exposure to inhaled chemicals as a new risk factor in acute pancreatitis (Segal 2000) and pancreatic cytochrome P450 induction in chronic pancreatitis (Norton 1997, Wacke 1998). In both scenarios GSH would potentially be involved in detoxification through conjugation.

5.2 Inefficiency of NAC / SAMe in acute pancreatitis.

If methionine metabolism is supported by the administration of exogenous transsulphuration intermediates, a benefit is undoubtedly gained against oxidant-induced tissue injury, Table 5.0. That such an avenue did not prove to be of benefit In our trial of NAC and SAMe in human AP, suggests that additional factors pervade and require addressing.

The differences between hepatocytes and acinar cells in man, help us explain why the NAC / SAMe combination seems to protect the liver against paracetamol but not the acinar cell in acute pancreatitis (Chapter 2). The new finding of profound blood folate deficiency (Chapter 3) at the time of admission with AP, in retrospect, offers an additional rational explanation, as does the marked ascorbate lack. Experimental studies show the effect that deficiencies of these two micronutrients exerts on exocytosis (Balaghi 1995, Nonaka 1991) and homeostasis of the methionine transsulphuration pathway, Figure 1.1.

Further studies to explore the therapeutic opportunities of antioxidant supplementation in human AP should comprise a combination of SAME / NAC with ascorbate, folate and selenium (Kuklinski 1993). Pyridoxine availability in AP has not been examined and should also be supplemented, unless appropriate studies are

able to confirm sufficiency. Vitamin B_{12} does not appear to be a limiting factor, although if oxidant load is prolonged or excessive may become so (Sharer 1983).

Antioxidant therapy, bolster methionine transsulphuration, aims to maximise GSH supply. The GSH precursor oxathizolidine-4-carboxylic acid (OTZ) normalises GSH levels and reduces inflammatory cytokine levels (TNF, IL-8 and IL-6) over a 9 day period, in alcoholic cirrhosis (Pena 1999). Thus, analogous to studies on the platelet activating factor (PAF) antagonist - Lexipafant, administration of antioxidant therapy should be extended to 72 hour or further, to fully assess its impact on pancreatitis in large scale trials (Kingsnorth 1995, Johnson 2001).

Supplement utilised	Mode of injury	Species	Organ examined	Reference
Methionine	Alcohol	Rat	Liver	Parlesak, 1998
GSH	Caerulein	Mouse	Pancreas	Neuschwander-Tetri, 1992
	t-butyl hydroperoxide	Rat	Lung	Brown, 1994
	CCl_4	Rat	Liver	Arosio, 1997
SAMe	CCl_4	Rat	Liver	Corrales 1992
	Ischaemia / transplantation	Pig	Pancreas	Scott, 1992
	Alcohol	Rat	Liver	García-Ruiz, 1995
	Alcohol	Man	Liver	Mato, 1999
NAC	Paracetamol	Man	Liver	Smilkstein, 1988; Harrison 1990; Keays, 1991
	ARDS	Man	Lung	Jepsen, 1992; Domenighetti 1999
	LPS	Rat	Lung	Davreux, 1997
	LPS Caerulein	Rat Mice	Lung Pancreas	Sprong, 1998; Demols, 2000

Table 5.0. Summary of studies in which supplementation of the transsulphuration pathway has proven to be of benefit in ameliorating oxidant-related organ damage.

[ARDS - adult respiratory distress syndrome, LPS - lipopolysaccaride].

5.3. Methionine / GSH lack as a conditioning but not precipitating factor in human acute pancreatitis.

Whereas the abrupt denial of methyl groups (by feeding a CDE diet) or GSH (by exposure to buthionine sulfoximine) is sufficient to precipitate acute pancreatitis in rodents (Lombardi 1976, Lüthen 1994c), gradual depletion mimics kwashiorkor, or chronic pancreatitis-like changes (Longnecker 1991, Véghelyi 1950). The Manchester studies of habitual diets in patients with idiopathic CP identified lower intakes of methionine and Vitamin C as being equally important factors when compared to diets of epilepsy patients with an equal degree of cytochrome P450 induction, while selenium status was equally poor in the two groups relative to controls (Rose 1986, Uden 1988a). Furthermore, plasma SAMe levels between exacerbations were lower than in controls (Schofield 1991, Uden 1992), as also was urinary sulphate excretion (Märtensson 1986a and Chapter 4) which endorse the dietary studies.

At the time of a pancreatitis attack, whether apparently 'acute' or an exacerbation of 'chronic,' and irrespective of the trigger (Roth 1985, Märtensson 1986a) an abrupt increase in methionine and SAMe, with a fall in GSH and energy charge (ATP) is identified (Schofield 1991). These changes are interpreted as evidence of functional inhibition to at least one or more points in the methionine transsulphuration pathway. Attempts to expose this vulnerability and pinpoint its site, whether impeding SAMe methylation, the recycling of homocysteine, the onward metabolism of cysteine and / or its incorporation with glycine into GSH, by exogenous loading proved unsuccessful. Given that all experimental protocols to generate AP involve a burst of free radical activity (Wallig 1998, Braganza 1991, 2000) the corollary to this is that methionine / GSH / SAMe lack is a contributing factor. In other words, acute oxidative stress will only trigger acute pancreatitis when sulphur amino acid supply is short or weak. This situation should perhaps have been anticipated from the studies of Véghelyi who suggested a far higher degree of pancreatitis when methionine deficient rats are exposed to carbon tetrachloride. The recognised vulnerability of the current studies is of the lack of data pertaining to the Vitamin B_6-dependant enzymes involved with the homocysteine to cysteine via cystathionine. These steps are very

relevant, as demonstrated in studies on the contraceptive pill (Steegers-Theunissen 1992) and in south Africans (Ubbink 1995).

5.4. Suggestions for future studies.

That the intervention with intravenous NAC and SAMe to patients with acute pancreatitis failed to demonstrate efficacy, and that elevation of plasma methionine was not confirmed in patients with quiescent recurrent pancreatitis, does not negate the ever increasing body of evidence that oxidative stress and interruption to methionine transsulphuration metabolism, are intricately and intimately linked with inflammatory pancreatic disease. The finding of a folate deficiency on admission in patients with acute pancreatitis supports an earlier dietary experimental model (Balaghi 1995). This opens up new avenues for therapeutic vitamin and micronutrient supplementation in pancreatitis to be incorporated into the combination already shown to be of clinical benefit (Uden 1990a, Sharer 1995, McCloy 1998).

Studying the transsulphuration pathway, Figure 1.3, points of vulnerability and mechanisms for raised homocysteine levels can be deduced. Low folate and / or Vitamin B_6 will manifest as hyperhomocysteinaemia, as will pernicious anaemia and other causes of a lack of Vitamin B_{12}. Hyperhomocysteinaemia is recognised as a risk factor in vascular disease, with acute elevations in its concentration resulting in endothelial dysfunction (Usui 1999). Premenopausal woman seem to metabolise proportionally more methionine by transamination, an alternative to transsulphuration that does not have homocysteine as one of its intermediate. As a result of this, the natural tendency is for pre-menopausal women to run lower homocysteine levels than men or post menopausal woman. This may be reflected in their reduced risk of premature vascular disease (Blom 1988). Urine samples that could be analysed for 4-methylthio-2-oxo-butyrate - an intermediate of the transamination pathway and deproteinised plasma for homocysteine analysis remain stored from the challenge study samples. Future works are planned to further examine methionine metabolism in pancreatitis by quantitating these analytes.

The mode of folate deficiency in AP requires exploration, for folate catabolism in not accelerated by alcohol-based enzyme induction (Kelly 1981), yet urinary folate excretion rate is increased by 20 - 40% with chronic alcohol ingestion (Russell 1983). Future studies to quantify urinary folate losses in acute pancreatitis is therefore indicated. In addition to a lack of folic acid, evidence that Vitamin B_6 deficiency impedes pancreatic acinar cell function (Singh 1980), and flux through the transsulphuration pathway exists (Davies 1986). Establishing Vitamin B_6 concentrations during the acute phase of pancreatitis would be informative, as would quantitation of cystathionine β-synthetase and cystathionase γ-lyase activity, both in experimental animal models and human disease, in particular seeking enzyme inactivation induced by oxidative stress.

Mitochondria, the site of oxidative phosphorylation, the main source of energy production, are as a consequence also the principle place of reactive oxygen species production. Chronic alcohol intakes reduces hepatocyte mitochondrial GSH, whereas restoration of GSH (by administration of SAMe, or glutathione ethyl ester) reverses the prior sensitisation of hepatocytes to the oxidative effects of cytokines, that occurs with GSH depletion (García-Ruiz 1995, Pena 1999). Blood GSH reflects intracellular levels in rodents (Dass 1992), and although this situation may not extend to humans (Purucker 1990), intracellular levels are tightly controlled in all species and maintained within strict limits. It is not unreasonable to suggest that it is likely that cells are unable to withstand anything other than brief periods of intracellular GSH insufficiency, for longer periods are incompatible with cellular integrity and are likely to result in cell death or apoptosis.

Choline and betaine each have methyl groups that may potentially feed via the transsulphuration pathway into SAMe, the universal methyl donor. Choline is also essential for phospholipid synthesis. These fatty acids are cyto-protective and have been shown to protect the rat pancreas from alcohol-induced oxidative stress (Aleynik 1999). It is well recognised that alcoholism results in hepatic steatosis as well as pancreatitis. Mechanisms that produce comparable hepatic changes might

also be expected to have the capacity to cause pancreatitis, through similar metabolic disturbances. Interruption of the choline-betaine supply underlies the CDE-dietary model of severe experimental AP. Thus it is not surprising that TPN-associated choline deficiency manifests as hepatic steatosis (Chawla 1989). Furthermore, intravenous choline administration (Buchman 1995) and oral betaine (Barak 1997), are capable of reversing or protecting against alcohol-induced fatty liver. Choline and Betaine levels were not recorded in this study, but do represent an area for future study.

Micronutrient supplementation comprising:

(i) *selenium* - to ensure amongst other functions that glutathione peroxidase is fully active,

(ii) *ascorbic acid* - to maximise GSSG reduction and to scavenge aqueous phase oxidants,

(iii) *folic acid* - to guarantee methyl group transfer and availability,

(iv) *methionine* - to prime the transsulphuration pathway;

is the most readily amenable manner in which to protect cells from oxidative stress. In this context it would be interesting to perform a double-blind randomised controlled trial of supplementation with Bioantox plus folic acid and Vitamin B_6 against placebo in patients following their first presentation with alcoholic and / or idiopathic (non-gallstone) acute pancreatitis. A significant reduction in the number progressing to a further attack of AP (RAP) or who develop CP would demonstrate efficacy.

Our understanding of the aetiology of pancreatitis has progressed in leaps and bounds over the past 20 years. No advance offers more promise than recent elucidation of the molecular basis to pancreatitis. The hereditary (familial) form of the disease has been shown to be due principally to mutations of the cationic trypsinogen gene (Whitcombe 1996) and possession of one or more mutations of the cystic fibrosis transmembrane regulator *(CFTR)* gene also predisposes to pancreatitis (Sharer 1998). It is surely no coincidence that both of these entities have been reported to

co-exist with oxidative stress and antioxidant deficiencies (Mathew 1996, Uden 1990b). A trial designed to examine the benefit of antioxidant supplementation in the hereditary form of the disease is already underway.

No longer is the pancreas the obscure, poorly understood, retroperitoneal organ it once was. The future is bright and it is not over optimistic to anticipate that the development of effective therapies and prophylactic measures to combat AP and CP will come to fruition within the next two decades.

APPENDIX A ANALYTICAL METHODOLOGIES

A1.0 Plasma Selenium

A1.1 Principle

Selenium-containing compounds (e.g. seleno-amino acids, glutathione peroxidase etc.) are oxidised in a strongly oxidising environment to Se (IV). The resulting Se (IV) is complexed with 2,3-diaminonaphthalene - a strong fluorophore - and the resulting complex is measured fluorometrically (MacPherson 1988).

A1.2. Reagents

a) Concentrated nitric acid (AnalaR, BDH). S.G. 1.42

b) Perchloric Acid (AnalaR, BDH). S.G. 1.70

c) 10% HCl:

- 100ml concentrated HCl (AnalaR, BDH). S.G. 1.18, 35%, made up to 1 litre in graduated flask (stable until used).

d) EDTA / HAC solution:

- 9g Na2EDTA.2H2O and 25g Hydroxyl Ammonium Chloride (AnalaR, BDH), made up to 1 litre with milli-Q water in graduated flask (stable until used).

e) 0.1M HCl:

- 8.73ml conc. HCl (AnalaR, BDH). S.G. 1.18, 35%, made up to 1 litre with milli-Q water in graduated flask (stable until used).

f) Diaminophthalene solution:

- 100 mg of 2,3-diaminophthalene (Aldrich) made up to 200ml in reagent e). Transfer to a 1L separating funnel and add 20 ml of reagent (g). Shake vigorously (release built-up gas). Allow to separate in dark. Repeat. Transfer diaminophthalene solution to a brown glass container; shield from light by wrapping container in tin foil. Cover with thin layer of (g). Store in dark, stable for 3 weeks at room temperature.

g) Cyclohexane (Aldrich, HPLC grade):

h) 0.1M NaOH

- 0.4g NaOH pellets (BDH, AnalaR) made up to 100 ml with milli-Q water in 100 ml graduated flask (stable until used).

i) **0.001M NaOH** made up to 100 ml with milli-Q water in 100 ml graduated flask (stable until used).

j) **Cresol Red Solution:**

- 20mg cresol red made up to 100 ml with solution (i) in 100 ml graduated flask (stable until used).

k) **40% Ammonia solution** (BDH, AnalaR).

l) **40% solution of 40% Ammonia solution**:

- 100 ml of solution (k) made up to 250 ml with milli-Q water in 250 ml graduated flask (stable until used).

m) **200μg/ml - Stock Selenium standard solution**

- 20 mg selenium (BDH, AnalaR)dissolved in 1 ml solution (a) and made up to 100 ml with milli-Q water in 100 ml graduated flask (stable until used).

n) **200ng/ml - Working Selenium standard solution**

- 0.1 ml of solution (a) and made up to 100 ml with milli-Q water in 100 ml graduated flask (stable 3 months).

o) **Hexane (Rathburn** (HPLC grade):

p) **Seronorm SNTE:**

- Commercially available standard serum solution containing 100 ng/ml selenium. Stable 4 years lyophilised, 1 month at -20oC reconstituted.

A1.3. Assay

- **NOTE** - all glassware should be washed with 10% nitric and dried prior to use in this assay.
- The evening before, select serum from 11 samples along with one 500 ml aliquot of **(p)**. Place 250 ml of each sample in a B14 stoppered tube in duplicate. Add 0 75 ml **(a)** to each tube. Replace glass stoppers.
- A transient precipitate is observed which redissolves within a few minutes. Leave to digest at room temperature overnight , in fume cupboard.

- First thing following morning, switch on the digestion block at maximum temperature. Add a few anti-bumping agents to each tube with 0.5 ml solution

(b). Mix. Allow digestion block to reach 285 - 300° C. Place each tube in a slot in the digestor, rotating gently to ensure good thermal contact via the sand in each slot. Within a few seconds, brown fumes will be evolved, lasting for approx. 3 - 4 minutes, replaced by fairly dense white fumes. This will be accompanied by fairly vigorous effervescence. Digest the samples for 45 minutes. When digestion is complete, the effervescence subsides and the samples should be colourless.

- Remove the tubes from the digestor, 4 at a time, and whilst still hot, with great care, add 0.5 ml of solution **(c)** to each of the 16 tubes.
- At this stage only white fumes should be evolved. If brown fumes evolve, then the assay in that particular tube has failed and will need repeating.
- Leave tubes to cool at room temperature.

A1.4. Standards

- Prepare a standard curve standards thus:

Tube no.	Volume solution (n) in µl.
S0	0
S10	50
S20	100
S30	150
S40	200
S50	250

- Add 0.5 ml solution **(b)** and then 0.5 ml solution **(c)** to each in turn. Mix.

A1.5. All Tubes

- Add 2.5ml of solution **(d)** and 4 drops **(j)** to each tube. Mix well.
- Titrate each tube to pH 2, using (l) to take the colour to primrose yellow and **(c)** to return to a peach-pink colour. Add 2.5ml of milli-Q water to each tube.

- **CLOSE LAB WITH BLACKOUT BLINDS AND USE RED SAFETY LIGHT**

- Add 2.5ml of solution **(F)** to each tube and place in water bath at 50^0 C for 30 minutes. Remove and cool to room temperature.
- Add 1 ml (g) to each and shake vigorously for 20 seconds.
- Allow to separate and transfer to fluorimeter tubes.
- **RETURN TO NORMAL LIGHT**

- With fluorimeter slits set at 15 nm (Ex) and 20 nm (Em), set wavelengths at 365 nm (Ex) and 515 nm (Em).

The flow cell is now flushed with **(o)** before the auto-sampler continues the analyses.

A1.6. Calculation

- Plot standard curve and calculate linear regression parameters, A, B and r. If a straight line exists for S0 and S50 then use linear regression values for samples. If curve read direct from chart.
- Selenium = ng in tubes x 4 = ng / ml = µg / l.

A2.0. Plasma Vitamin C

A2.1 Principle

The spectrophotometric technique method of Omaye 1979 is based on the reactions:-

Ascorbic Acid + Cu $\xrightarrow{\text{oxidation}}$ Dehydroascorbic and Diketogulonic acids

Add 2,4, *dinitrophenylhydrazine* $\longrightarrow$ bis 2.4-DNPH

Add H_2SO_4 $\xrightarrow{\text{rearrangement}}$ Red colour.

The reaction is carried out in the presence of thiourea (a mildly reducing substance) to provide optimal conditions for detecting ascorbic acid without interference from other chromogens.

A2.2. Reagents

- Trichloroacetic Acid 5% aqueous for preparation of standards and 10% aq. for treatment of plasma samples, keep in fridge.
- Stock thiourea solution, 657mM aq. = 0.500g / 10ml.
- Stock $CuSO_4$ solution, 24mM aq. = 0.060g / 10ml.
- Stock 2,4, dinitrophenylhydrazine solution, 111mM in 5M H_2SO_4 = 0.4399g / 20ml. (N.B. because of the explosive nature of 2,4-DNPH, it is usually supplied with added moisture. The amount added is stated on bottle. Allow for this when weighing e.g. often x 4/3. It is toxic by inhalation and skin contact, therefore full protective precautions with use of gloves, goggles and fume extractor cabinet are necessary).
- Ready prepared BDH 65% H_2SO_4 is kept ice cold in fridge.
- Working DTC reagent, mixed freshly as required, and may be kept for 1 week at 4^{o} C: Add

  0.5 ml thiourea stock solution

  0.5 ml $CuSO_4$ stock solution

  10.0 ml 2,4-DNPH stock solution.

A2.3. Equipment

Spectrophotometer (Cecil CE515) 520nm

Constant temperature waterbath 37° C

A2.4. Procedure

Standards

- Prepare a range of 0 - 15 mg/l ascorbic acid in 5% TCA thus:- weigh out 200mg / 1000ml in 5% TCA and dilute x 100 with 5% TCA giving 20mg / L.

Vitamin C final conc. mg L	Vitamin C (20mg / L) ml	5% TCA ml
0	0	4.0
2.5	0.5	3.5
5	1.0	3.0
7.5	1.5	2.5
10.0	2.0	2.0
12.5	2.5	1.5
15.0	3.0	1.0

Samples

- Heparinised blood is collected and centrifuged at 1000g (2000 r.p.m) for 10 - 15 minutes.
- 2.4ml of plasma is then added to an equal volume of 10% TCA.
- Mix by vortex and stand for 5 minutes before mixing once more.
- Centrifuged at 3000g (4500 r.p.m) for 20 minutes.
- Separate off supernatant and freeze at -70^{O}C until analysis.

All Tubes

- Take 2 x 10ml screw top tubes (duplicates) for each standard or sample.
- Mix well all samples or standards that have been frozen prior to analysis.
- Pipette 1.33ml of sample or standard into appropriate tube and 1.33ml 5% TCA for blank.
- Add 0.4ml DTC reagent to all tubes. Replace screw caps and mix well.

Incubate at 37^{O}C for 4 hours, mixing periodically. Then place tubes on ice to cool.

- Whilst still on ice, add 2.0ml ice-cold 65% H_2SO_4 to each tube. Replace caps and mix well - H_2SO_4 is quite viscous. Stand for 30 minutes at room temperature for red colour to develop.
- Read absorbance of all tubes at 520nm against the TCA blank.

A2.5. Calculation

When plotted the standards should give a linear or slightly curved calibration line. The vitamin C concentration can be read off this and the result multiplied by 2 (to correct for initial dilution) to give final value.

Normal range is 4 - 20 mg / L (23 - 114 μM/l).

A2.6. Quality Assurance

Accuracy -

If any calculated sample concentration exceeded 20 mg / L, the analysis was repeated in a subsequent run, diluting the sample 1:1 with 5% TCA.

A3.0. HPLC Assay for Plasma Ascorbic Acid (and Dehydroascorbic acid)

A3.1. Principle: Plasma ascorbic acid (AA) and uric acid (UA) are quantified by HPLC with UV detection (Schofield 1990). The sample is the reduced with homocysteine to convert any dehydroascorbic acid (DHAA) present to AA, then re-assayed. The DHAA level is calculated by subtraction of the intrinsic AA concentration.

A3.2. Reagents

- Metaphosphoric acid (MPA) 10% solution w/v. made up with Milli-Q water.
- Homocysteine (Sigma) 1mM, {34mg in 250ml Milli-Q water}.
- Ascorbic acid standard ca. 20µg/ml, {20mg (BDH, Analar) made up to 100ml with homocysteine 1mM. Take 0.5ml of this and dilute 1:10 with homocysteine 1mM. Make fresh daily.
- Uric acid (Sigma) 450 µM standard in Milli-Q water.
- K_2HPO_4 solution 45% w/v. {3.6g $K_2HPO_4.3H_2O$ (BDH, Analar) and 8 ml filtered Milli-Q water in 10 ml screw cap tube, vortex to dissolve}.
- Homocysteine (1% in K_2HPO_4 for reduction). Vortex mix homocysteine 30mg and K_2HPO_4 solution 3ml. Centrifuge at 3000rpm pat 4°C prior to use if cloudy. Make fresh daily.

A3.3. Procedure

Standards

Two standard curves are prepared n parallel:-

1) AA / UA in acidic solution (MPA) - for AA determination.
2) AA / UA in neutral solution, for determination of AA and DHAA.

The following mixtures are prepared and run prior to any plasma samples to check resolution and response are satisfactory. During analysis run it is usually necessary to re-run some acidic and neutral standards to optimise resolution and confirm assignment of peaks.

Standard curve:

Vial no.	AA (µl)	UA (µl)	1mM Homocysteine (µl)	MPA (µl)	H_2O (µl)
1	-	-	500	500	-
2	400	100	-	500	-
3	400	100	-	-	500
4	300	200	-	500	-
5	300	200	-	-	500
6	200	300	-	500	-
7	200	300	-	-	500
8	100	400	-	500	-
9	100	400	-	-	500

Samples

- Heparinised blood is collected and centrifuged at 700g (2000 r.p.m) for 10 - 15 minutes.
- 0.5ml of plasma is then added to an equal volume of 10% TCA.
- Mix by vortex and stand for 5 minutes before mixing once more.
- Centrifuged at 3000g (2000 r.p.m) for 10 minutes.
- Separate off supernatant and freeze at -70^{O}C until analysis.
- Prior to use, thaw and mix well. If sample is cloudy centrifuge at 3000rpm for 10mins.

HPLC conditions

a) Pre-column: Co-Pell ODS (40µm) 50 x 4.6 mm i.d.
Column: Apex AminoRP 5µ 250 x 4.6 i.d.

b) Mobile phase: Make up 6.805g K_2HPO_4 to 1 litre with Milli-Q water. Check pH 4.5 - 4.55. Filter buffer through Nylaflo filter 0.45µm, then transfer to Winchester in water bath at 40^{o}C to equilibrate. Add 1500ml filtered S-grade acetonitrile in portions over 30 minutes. Sonicate for 20 minutes before use.

Equilibrate column with 1 litre eluant and run at 0.2ml/min overnight when not in use to prevent algae growth. Run eluant and 1.5ml/min for 1 hour before first injection.

c) Detection: Waters 490 dual channel UV detector set to 254 and 270nm.

d) Integrator: Spectre Physics SP4290 wth memory module and channel B. Chart speed = 0.25, Attenuation = 32 (both channels).

e) Auto sampler: Kontron MS1660T, loop size 20µl.

Sample procedure.

Pipette 600µl MPA deproteinised plasma into a 2ml autosampler vial and insert into cooled autosampler. After checking resolution of AA peak is satisfactory, run an acidic or neutral standard as appropriate. Then pipette 400µl remaining sample into a second vial, add 200µl 1% homocysteine solution dropwise with vortexing to avoid local increases in pH which might result in hydrolysis. Allow sample to stand for 15 minutes at room temperature in dark for complete reduction , then vortex again and place in autosampler.

A3.4. Results

- Check peak ratio 254:270 is ~ 1 - 1.3 for UA and 2 for AA
- Retention times vary but are typically ~ 5.5mins for UA and 6.3mins for AA.

A3.5. Calculations

- Plot standard peak area against volume of standard used, in µl for both acidic and neutral standards. Should be linear and pass through origin.
- Calculate equivalent volume for real samples by linear regression.
- Then: 1µl UA = 0.9µM in acidic samples
  1µl UA = 1.35µM in reduced samples
  1µl AA = A/500µg/µl in acidic samples, where A is initial mass of AA weighed.
  1µl AA = 1.5A/500µg/µl in reduced samples.

A3.6. Normal range

- UA 110 - 450μM
- AA 4.5 - 18μg/ml
-

A3.7. Quality control

Within batch Cv. = 4.7% for AA
= 6.1% for AA + DHAA
= 2.2% for UA

Between batch Cv. = 6.8% for AA
= 6.5% for AA + DHAA
= 3.1% for UA

Recovery of AA added to plasma prior to deproteinisation (intrinsic level = 13.9μl/ml)

Added AA (μg/ml)	Recovery % AA	Recovery % AA + DHAA
4.62	78	91
9.25	92	86
13.87	98	107.

A4.0. Analysis of Fat Soluble Vitamins - (β-Carotene, Retinol and α-Tocopherol).

A4.1. Principle

The HPLC analysis of fat soluble vitamins is based on the method described by Thurnham 1988.

A4.2. Standard solution preparation:

Prepare in dark, using only safelight. Cover volumetric flask with aluminum foil.

Standards from Sigma: (store at -20°C.)

α–Carotene,	Type V	C.0251	1mg
β-Carotene,	Type IV	C.0126	5mg
Retinol,		R.7632	25mg
Tocopherol	dl-a-	T.3251	5g
Tocopherol acetate	dl-a-	T.3376	5g

Standard Stock Solutions: (store at -20°C.)

α–Carotene,	1mg	in	10ml	hexane / BHT
β-Carotene,	5mg	in	25ml	hexane / BHT
Retinol,	25mg	in	100ml	absolute alcohol
Tocopherol	5g	in	100ml	absolute alcohol
Tocopherol acetate	5g	in	100ml	absolute alcohol

Working Solutions: (store at 4°C, ~ 1 week)

			Theoretical concentration
α–Carotene,	100µl	10ml	1 mg / L
β-Carotene,	100µl	10ml	2 mg / L
Retinol,	100µl	10ml	2.5 mg / L
Tocopherol	100µl	10ml	0.1 g / L
Tocopherol acetate (calibration solution)	3ml	20ml	~ 0.1 g / L

Internal standard solution:- dilute calibration solution x 5.

N.B. During storage, the standard solutions may precipitate out to varying degrees, and will not be at the theoretical concentrations calculated above. Therefore when the working solutions have been prepared, the absorbance is measured at an appropriate wavelength and compared with known absorbances.

Calculation of exact concentration of working solutions:

The absorbances which should be given by the working solutions can be calculated as follows:

	λ (nm)		E1%	Theoretical concentration	Desired O.D.
α–Carotene,	446		2710	1 mg / L	0.271
β-Carotene,	452	in	2550	2 mg / L	0.510
Retinol,	325	in	1780	2.736 mg L	0.487
Tocopherol	292	in	75.8	0.1 g / L	0.758
Tocopherol acetate	285	in	43.6	0.1 g / L	0.436

If the observed OD is near the desired OD, the observed OD is noted and used to calculate the actual concentration. However, if the observed OD is not near enough to the desired OD, the concentration is adjusted by adding more stock solution or solvent as necessary, and the new OD is recorded.

Purity checks

Each time the working solutions are prepared it is necessary to check their purity by running each separately on the HPLC at the above wavelengths. The purity is then calculated from the areas of all the peaks present for each standard.

The concentrations calculated from the OD values above are then further adjusted to take account of purity.

Example of purity calculation:

For β-Carotene at 452 nm. OD of 0.510 = 2 mg / L

Observed OD = 0.528

Total area of peaks on HPLC trace at 452 nm. = 326,000.

Area of β-Carotene peak = 301,876. Purity = 92.6%

Actual concentration of β-Carotene in working solution:-

= 2 x 0.528 / 0.510 x 92.6 / 100 = **1.917 mg / L**.

A4.3. Standards preparation:

Standard solutions should be kept on ice when not actually in use. All preparation should be performed in the dark with only a safelight if possible.

The following standard mixtures are prepared by pipetting the amounts of working solutions shown below, blowing to dryness with O2-free nitrogen and redissolved in the mobile phase. The tubes are held in a beaker of water at room temperature whilst blowing to speed up the evaporation. When redissolved, transfer the solutions to the vials in cooled autosampler.

Standard no.	α– Carotene	β- Carotene	Retinol	Tocopherol	Calibration solution	Internal Standard	Redissolve in MP
S1	100µl	–	–	–	400µl	–	1ml
S2	–	100µl	–	–	400µl	–	1ml
S3	100µl	100µl	–	–	400µl	–	1ml
S4	100µl	100µl	200µl	100µl	400µl	–	1ml
S5	–	–	–	–	–	500µl	200µl

These 5 standard solutions are run each day at the beginning of the run. The mixed standard, S4, used for calculating sample results, is also moved along the carousel and run every fourth sample. Standard S1 - S3 are not used for calculating the sample results, but are run to check the separation of α- and β-Carotene. Standard

S5 is run to check on the recovery of the internal standard at the extraction step of sample preparation.

For purity checks, the following tubes are prepared in a similar manner:-

Tube	α–Carotene	β-Carotene	Retinol	Tocopherol	Redissolve in MP
S1	100µl	–	–	–	1ml
S2	–	100µl	–	–	1ml
S3	–	–	200µl	–	1ml
S4	–	–		100µl	1ml

HPLC Conditions

Column: LKB Superpac cartridge column.
Spherisorb ODS2, 3µ, 4 x 100 mm, used with
Precolumn filter, Anachem A-316, 0.5µ frits.

Mobile phase: Methanol : Acetonitrile : Chloroform
47 : 47 : 6.

Flow rate: ~ 1ml / minute (pressure 50 - 60 bar).

Detector: Waters 490 uv / vis multichannel
Channel (1) 450 nm AUFS 0.4 for α- and β-Carotene
Channel (2) to 325 nm AUFS 1.0 for retinol
$t_{2.5}$ 292nm AUFS 1.0 for tocophol (acetate).

A4.4. Sample preparation:

Serum samples will have been stored at - 70°C and not previously been thawed and re-frozen. Add the following into glass centrifuge tubes:-

i) 250µl serum with pipette.

ii) 250µl SDS solution and vortex for 1 minute.

iii) 500µl internal standard solution. Vortex for further 1 minute (will precipitate protein).

iv) 1000µl hexane / butylated hydroxytoluene. Vortex for 1 minute and then centrifuged at 3000 rpm for 10 minutes.

Then carefully remove 700µl of upper (hexane) layer into a clean tube. Blow to dryness under nitrogen as for standards. When dry redissolve by Vortexing in 200µl mobile phase. Then transfer solution to auto-sampler vials.

A4.5. Vitamin concentration calculation

Standard curves are constructed for each of the fat soluble vitamins from the calibration curves, corrected for purity. The peaks areas of each vitamin of interest are then read off this curve and the data stored on a *Microsoft Excel* file for future analysis.

A5.0. Glutathione

A5.1. Principles

Whole blood and plasma were analysed for total glutathione (including oxidised and reduced forms), by spectrophotometric recycling method utilising a kinetic enzyme assay involving glutathione reductase / NADPH couple as described by Schofield 1993.

A5.2. Glutathione assay reagent preparation.

All solutions are prepared in HPLC grade (with Milli-Q water).

Buffer 1: (Potassium phosphate 100mM, Na2EDTA 5mM, pH 7.50) - Make up KH2PO4 (1.361g, MW = 136.09, BDH AnalaR) and Na2EDTA (186.1mg, MW = 372.24, BDH AnalaR) to 100ml - "K1 buffer". Make up K2HPO4.3H2O (4.565g, MW = 228.23, BDH AnalaR) and Na2EDTA (372.2mg MW = 372.24, Na2EDTA)to 200 ml - "K2 buffer". Add "K1" buffer to approx. 50ml "K2" buffer in proportions until pH = 7.50 - should require approx. 30ml. Buffer replaced weekly.

DTNB buffer: (Potassium phosphate 100mM, Na2EDTA 17.5 mM, pH 7.50) - Make up KH2PO4 (1.361g, MW = 136.09, BDH AnalaR) and Na2EDTA (651.4mg, MW = 372.24, BDH AnalaR) to 100ml - "K1" buffer." Make up K2HPO4.3H2O (2.282g, MW = 228.23, BDH AnalaR) and Na2EDTA (651.4mg MW = 372.24) to 100ml - "K2" buffer." Add "K1" buffer." To approx. 50ml "K2" buffer" in proportions until pH = 7.50. Should require less than 1ml. Replaced weekly.

5,5'-Dithiosbis(2-nitrobenzoic acid) (DTNB) (10mM solution in DTNB buffer) - weigh approx. 20mg DTNB (MW =- 396.36, BDF) to 0.1mg precision in a 10ml screw-capped tube, add the appropriate volume of DTNB buffer, and vortex-mix until dissolved - may take several minutes. Replace weekly and store at 4^{o}C.

NEM Buffer (Potassium phosphate 100mM, Na2EDTA 17.5 mM, pH 6.50) - take remaining 50ml "DTNB buffer "K2" buffer" in proportions until pH = 6.50 - should require approx. 70ml. Replace buffer weekly.

NEM (10mM solution in NEM buffer) - Weigh N-ethylmaleimide (NEM) (ca. 12mg, MW = 125.1, Sigma) to 0.1mg precision in a narrow-capped 10ml screw-capped tube. Add the appropriate volume of NEM buffer to give a 10mM solution and dissolve by vortexing. Store at store at 4°C and replace weekly.

Haemolysis Buffer (Sodium phosphate 10mM, Na_2EDTA 0.5mM, pH 7.40) - Make up NaH2PO4.2H2O (156.0mg, MW = 156.01, BDH AnalaR) and Na2EDTA (18.6mg, MW = 372.24, BDH AnalaR) to 100ml - "Na1 buffer". Make up Na2HPO4 (142.0mg, MW = 141.96, BDH AnalaR) and Na2EDTA (18.6mg, MW = 372.24, BDH AnalaR) to 100 ml - - "Na2 buffer". Add - "Na1 buffer" to all - "Na2 buffer" in proportion until pH = 7.4 - should require approx. 25ml. Replace weekly.

Phosphate Buffered Saline (PBS) - dissolve 1 tablet PBS (Dulbecco 'A' Oxoid) in Milli-Q water, and make up to 100 ml. Replace weekly.

Oxidised Glutathione (GSSG) - Stock standard (1mM in PBS) - weigh accurately approx. 2mg GSSG (MW = 612.6, Sigma) using Cahn balance, into a amber coloured screw-capped bottle. Make up to 1mM by adding an appropriate volume of PBS. Store at store at 4°C and replace weekly.

GSSG: Working Standards - were prepared daily and kept on ice. Dilution of stock solution as follows:-

i) 40µl 1mM stock + 960µl PBS → 40µM GSSG.

ii) 300µl 40µM GSSG + 2700µl PBS → 4µM GSSG.

iii) 500µM 4µM GSSG + 1500µl PBS → 1µM GSSG - for GSH / NEM determination only.

NADPH - (2.2mM in buffer 1) - Made up fresh on day of assay. Calculate effective MW of NADPH (type 1, tetrasodium salt, Sigma), allowing for water, ethanol and impurities - typically MW = 960, and thus, approximate quantity required - 220nmol per cuvette. Weigh NADPH onto small piece of aluminum foil to 0.1mg precision, and transfer to an amber glass screw-capped bottle (solution light sensitive).

Reweigh foil and dilute solution to 2.2mM (i.e. 220nmol in 100µl for assay), with Buffer 1.

Glutathione Reductase (GR) - (0.5 units in 100µl Buffer 1). Make up sufficient for the assays being performed on that day, and store on ice. Vortex mix GR suspension (usually 100 units in 0.5ml, Sigma, Type III - adjust volumes appropriately if different). Pipette 2.5µl per cuvette into 97.5µl Buffer 1 and mix.

Plasma Haemoglobin Assay - Sodium Carbonate - stock solution. - 1g (MW = 105.99, Hopkins & Williams, AnalaR) diluted to 100ml. Working solution: 1ml stock solution diluted to 100ml with Milli-Q water. Shelf life 3 months.

A5.3. Glutathione sample preparation

Prior to taking blood the following 1.5 ml Eppendorfs are prepared. These were made up on a weekly basis and store at 4°C until required:

a) 700µl NEM solution - labelled N - to be analysed for plasma GSSG.
b) Empty Eppendorf - labelled P - for plasma haemoglobin.
c) 200 µl DNTB solution - labelled D - for plasma GSH + GSSG.
d) 950 µl Haemolysis buffer stored on ice- labelled H - for whole blood GSH + GSSG.

Before blood is drawn the sample preparation Eppendorfs are stabilised on ice for near patient analysis. Gilson pipettes are set at the required volumes and blood taken with minimal stasis a stasis as is feasible, usually from an ante-cubital vein. Four mls freshly drawn blood is transferred to an EDTA tube and mixed by inversion. The following volume are then transferred to the Eppendorfs as follows:

a) Tube N - 700µl - ***This must be done within 120 seconds of drawing blood.*** mix by inverting twice.
b) Tube P - 700µl
c) Tube D - 200µl - mix by inverting twice.
d) Tube H - 50µl - left on ice for 5 minutes for cell lysis to occur.

Tubes N, P and D are then spun together at 2900g (6500 rpm) for 6 minutes. The supernatants are then transferred to new Eppendorfs and stored at - 70°C for further analysis.

After lysis, 50µl is transferred from Tube H to another tube H (containing a further 950µl of Haemolysate). The sample is mixed by vortexing and stored at - 70°C for further analysis.

The remainder of the blood in the EDTA tube is then labelled correctly with patients identification number, date of birth and time and date of collection and sent to the Haematology department for whole blood haemoglobin and haematocrit estimation.

A5.4. Plasma GSH +/- GSSG assay (tube D)

Assay 5 standards and up to 5 samples in duplicate a single run. The following GSSG standards are made from 4µM working stock in 1.5 ml Eppendorfs:-

µl GSSG	µl PBS	µl DTNB	[GSSG]/µM
0	400	400	0
100	300	400	1
200	200	400	2
300	100	400	3
400	0	400	4

N.B. The true GSSG concentrations for the above standards are ½ of those actually shown, but as the blood sample was originally diluted by a 1 : 2 factor in DTNB this allows the actual blood concentration to be read directly from the calibration curve.

Five cuvettes for the standards plus 2 for each sample (in duplicates) are prepared, each containing 750µl Buffer 1. To this is added 100µl Glutathione Reductase (GR) to each cuvette and allow to equilibrate at room temperature. *The remainder of the assay is performed under subdued lighting.*

In batches of five, 50µl of standard or DTNB treated plasma are added to the cuvettes and mix using a 1ml Gilson. With strict timing, at 1 minute intervals, 100µl NADPH is added and the contents mix as before with the Gilson. Absorbance at O.D. λ=412nm versus a Milli-Q water blank, at 0.5 and 30.5 minutes is measured to calculate the increase in O.D. λ=412nm, Δ O.D.$_G$.

Plasma contains substances which inhibit the Glutathione Reductase recycling reaction. The increase in O.D. λ=412nm is therefore corrected using an internal standard, as follows:- At 1 minute intervals 12.5µl of 4µM GSSG is added to each sample cuvette, using a positive displacement pipette. The reaction sample is mix and absorbance read at O.D. λ=412nm at 0.5 and 10.5 minutes. Δ O.D.$_S$ is calculate for the increase observed in this period.

A5.5. Calculations

Reaction rate for unknown sample (over 30 minutes) = Δ O.D.$_G$

Reaction rate for spiked sample (over 10 minutes) = Δ O.D.$_S$

:- Reaction rate for spiked sample (over 30 minutes) = 3Δ O.D.$_S$

:- Contribution from added 50pmol GSSG = "$\Delta\Delta$" = 3Δ O.D.$_S$ - Δ O.D.$_G$.

The standard curve for 0 - 4 mM GSSG is usually linear ($r > 0.998$). Hence calculate the intercept a) and gradient b) by linear regression. (typically a~0.10 and b~0.17).

Then uncorrected sample [GSSG] / µM = V_G = (Δ O.D.$_G$-a) / b.

Corrected sample [GSSG] / µM = V_c = (2Δ O.D.$_G$ / $\Delta\Delta$ - a / b.

These values would apply if plasma were treated initially with DTNB. However, whole blood is utilised and thus these values must be corrected for the haematocrit, H., by using the formula below. Moreover, values are quoted in GSH equivalents, (i.e. double the GSH concentrations).

:- Corrected plasma [GSH] / μM = V_c (2 - H) / (1 - H).

The degree of enzyme inhibition can be calculated from ratio R = ΔΔ / 2b. For no inhibition, R = 1. Typically for plasma samples 0.7 < R < 0.9.

A5.6. Plasma GSSG assay

Sample extraction.

Assay 5 standards and up to 5 duplicate samples in a single run. The following GSSG standards are made from 1μM working stock as follows in 1.5ml Eppendorfs:

μl GSSG	μl PBS	μl NEM	[GSSG]/μM
0	400	400	0
100	300	400	0.25
200	200	400	0.50
300	100	400	0.75
400	0	400	1.00

N.B. True GSSG concentrations for the above table are ½ of those actually shown, but as the sample was originally diluted by a 1 : 2 factor in NEM this allows the actual concentration to be read directly from the calibration curve. Vortex mix and store standard on ice. Spin down briefly all Eppendorfs containing NEM to minimise spillage.

NEM is removed from sample (N) and standards using BondElut C_{18} columns, which can be reused several times after regeneration as described below. The columns are numbered and any column which fails to perform adequately in two successive experiments is discarded.

The VacElut system is set up with as many as 10 numbered BondElut C_{18} columns. The columns are washed in turn with 3ml HiPerSolv methanol the 3ml Milli-Q water. The collection tube rack is the fitted and 250μl standard or thawed sample applied to each column - the samples are done in duplicate. A duplicate blank is

extracted for precision in standard curve intercept, otherwise standards are performed singly. A slight vacuum is required to pull the solution onto the stationary phase, but flow rate should be only approx. 1 drop per second to ensure all NEM is removed. GSSG is then eluted with 1 ml Buffer 1 - again at approx. 1 drop per second, and the sample rack then removed.

Column regeneration:-

The columns are washed in turn, under vacuum, with the following:-

1. 3 x 3ml Milli-Q water.
2. 2 x 3ml Solution D (MeOH/H_2O/HOAc 67:33:04).
3. 2 x 3ml Solution C (i-Propanol/Acetonitrile 2:1).

A5.7. Assay Procedure

Glutathione Reductase and NADPH solutions are prepared as described under reagents and an Eppendorf containing 50 : 50 600µl of both DTNB and PBS.

Six cuvettes for the standards plus 2 for each sample (in duplicates) are prepared, each containing 750µl BondElut column extract (after mixing with Gilson) into each cuvette. To this is added 100µl Glutathione Reductase (GR) to each cuvette and allow to equilibrate at room temperature. *The remainder of the assay is performed under subdued lighting.*

In batches of five, 50µl of 1 : 1 DTNB : PBS plasma to the cuvettes and mix using a 1ml Gilson. With strict timing, at 1 minute intervals, 100µl NADPH is added and the contents mix as before with the Gilson. Absorbance at O.D. λ=412nm versus a Milli-Q water blank, at 0.5 and 30.5 minutes is measured to calculate the increase in O.D. λ=412nm, Δ $O.D._G$.

Plasma contains substances which inhibit the Glutathione Reductase catalysed recycling reaction. The increase in $O.D_\lambda$=412nm is therefore corrected using an internal standard, as follows:- At 1 minute intervals 12.5µl of 4µM GSSG is added to each sample cuvette, using a positive displacement pipette. The reaction sample is

mix and absorbance read at $O.D_{\lambda}$=412nm at 0.5 and 10.5 minutes. Δ $O.D._S$ is calculated for the increase observed in this period.

A5.8. Calculations for plasma GSH

Reaction rate for unknown sample (over 30 minutes) = Δ $O.D._G$

Reaction rate for spiked sample (over 10 minutes) = Δ $O.D._S$

:- Reaction rate for spiked sample (over 30 minutes) = 3Δ $O.D._S$

:- Contribution from added 50pmol GSSG = "$\Delta\Delta$" = 3Δ $O.D._S$ - Δ $O.D._G$.

The standard curve for 0 - 1 μM GSSG is usually linear ($r > 0.998$). Hence calculate the intercept a) and gradient b) by linear regression. (typically a~0.10 and b~0.6).

Then uncorrected sample [GSSG] / μM = V_G = (Δ $O.D._G$-a) / b.

Corrected sample [GSSG] / μM = V_c = (2Δ $O.D._G$ / $3\Delta\Delta$ - a / b.

These values would apply if plasma were treated initially with DTNB. However, whole blood is utilised and thus these values must be corrected for the haematocrit, H., by using the formula below. Moreover, values are quoted in GSH equivalents, (i.e. double the GSH concentrations).

:- Corrected plasma [GSH] / μM = V_c (2 - H) / (1 - H) in GSH equivalents.

The degree of enzyme inhibition can be calculated from ratio R = $3\Delta\Delta$ / 2b. For no inhibition, R = 1.

A5.9. Whole blood GSH + GSSG (Tietze's method)

Assay 5 standards and up to 7 samples in duplicate a single run. The following GSSG standards are made from 4μM working stock in 1.5 ml Eppendorfs:-

µl GSSG	µl PBS	µl DTNB	[GSSG]/µM
0	400	400	0
100	300	400	1
200	200	400	2
300	100	400	3
400	0	400	4

N.B. The true GSSG concentrations for the above standards are ½ of those actually shown, but as the blood sample was originally diluted by a 1 : 2 factor in DTNB this allows the actual blood concentration to be read directly from the calibration curve.

Five cuvettes for the standards plus 2 for each sample (in duplicates) are prepared, each containing 750µl Buffer 1. Thaw samples in Tube H, vortex mix to ensure homogeneity, then add 25µl haemolysate to each sample cuvette. Add 100µl Glutathione Reductase (GR) to each cuvette and allow to equilibrate at room temperature. *The remainder of the assay is performed under subdued lighting.*

In batches of five, add 50µl of standard / DTNB (for standard cuvettes) or 25µl DTNB solution (for samples) to the cuvettes and mix using a 1ml Gilson. With strict timing, at 1 minute intervals, 100µl NADPH is added and the contents mix as before with the Gilson. Absorbance at O.D. λ=412nm versus a Milli-Q water blank, at 0.5 and 30.5 minutes is measured to calculate the increase in O.D. λ=412nm, Δ O.D.$_G$.

A5.10. Calculation for whole blood GSH + GSSG

Reaction rate for unknown sample (over 30 minutes) = Δ O.D.$_G$

The standard curve for 0 - 4 µM GSSG is usually linear ($r > 0.998$). Hence calculate the intercept a) and gradient b) by linear regression. (typically a~0.10 and b~0.17).

Then haemolysed sample [GSSG] / µM = $V_G = (\Delta$ O.D.$_G$-a) / b.

Values are quoted in GSH equivalents, (i.e. double GSSG concentrations), so correcting for haemolysis dilution we obtain:

Corrected whole blood total Glutathione [GSH] / μM =800 x V_G.

To allow for the variations in red cell mass between patients, whole blood haemoglobin and haematocrit are also determined by haematology. Results are then also calculated and expressed as:

1) μmoles per gram haemoglobin.

2) μmoles per litre red blood cells.

A5.11. Plasma haemoglobin

Prepare disposable cuvettes with 1ml working sodium bicarbonate solution in duplicate for each sample, plus one cuvette as a blank.

Thaw plasma samples, Tube P, and vortex mix to ensure homogeneity. To each sample cuvette add 100μl plasma and mix carefully with 1ml Gilson.

Set spectrophotometer to zero at 380nm with blank, and read $O.D_{\lambda}$=380nm relative to this blank. Repeat this procedure for $O.D_{\lambda}$=415nm and $O.D_{\lambda}$=450nm.

A5.12. Calculations for plasma haemoglobin

Plasma haemoglobin = 83.6 x (2 x $O.D_{\lambda}$=415nm - $O.D_{\lambda}$=380nm - $O.D_{\lambda}$=450nm) mg / dl. If duplicates are not consistent to within 1 mg /dl the sample duplicates are repeated.

A6.0. 9,11 / 9,12 Linoleic Acid Percentage Molar Ratio (%MR)

A6.1. Principles

The 9-cis,11-trans isomer of linoleic acid, expressed as a molar ratio of the parent fatty acid in serum was used as a marker of free radical-mediated attack on polyunsaturated fatty acids (lipid isomerisation). The HPLC separation is based on the methodology described by Iverson 1985 and Smith 1991.

A6.2. %MR assay reagents.

a) Phospholipase A_2 solution (PA_2).

To 1.2114g Tris add 50 ml water and 4.04ml methanol. Add 500u PA_2 (Sigma P 6139) and make up to 100ml with water. Adjust to pH 8.9 with N or N/2 HCl.

b) 0.5% Acetic acid in Methanol.

c) Isopropanol / Acetonitrile (2 : 1).

d) Methanol / Acetic acid / Water (67 : 0.04 : 33)

e) 9,11-Linoleic acid (trans-trans) stock internal standard, 1.5mg / ml Methanol.

f) 9,12-Linoleic acid stock standard, 100mg / 10ml Methanol.

g) HPLC mobile phase: Acetonitrile : Water / Acetic acid (900 : 100 : 1).

- Prepare internal standard in solution b) to final concentration 5mg/2ml (solution BS).

A6.3. Standard curve for 9,12-LA.

The standards for serum analysis make up to final volume 0.5ml:

Stock µl	MeOH µl	9,12-LA in assay µg
0	500	0
50	450	50
100	400	100
150	350	150
200	300	300

A6.4. Preparation of samples

Mix 0.5ml sample with equal volume solution (a).

Incubate at 25°C for 15 minutes.

For serum then add 1ml (BS) + 1ml (B), mix well and centrifuge 3,000 rpm for 15 minutes.

Prepare Bond-Elut column for separation by:

i) wash with 2 x 2.5ml solution (c).

ii) Immediately before use wash with 2 x 2.5ml solution (d).

Then add 1ml supernatant from reaction tube to column, wash column with 2 x 2.5ml solution (d).

Elute sample with 1ml solution (c).

Vortex collection tubes, store at -20 °C in autosampler vials until separated.

A6.5. HPLC conditions.

a) Column ODS C18 RP 4mm x 30cm (10μ)

Pre-column ODS C18 (5 or 10μ)

or Co-Pell ODS (40μ)

b) Mobile phase, solution (G). Flow rte 1.3 - 1.5 ml/min

c) Detection: UV - 2 detectors in series

i) 234nm Erma ERC-7210 range 0.04

ii) 205nm Cecil CE212 range 0.5

d) Integrator- SP 4290, with:

i) chart speed 0.25

ii) attenuation 4

iii) pH 1

e) Autosampler.

A6.6. Results

i) ***234nm*** (conjugated dienes) 9,11-LA - Retention time 7.7 mins

Int. Std - Retention time 8.8 mins

ii) ***205nm*** 9,12-LA - Retention time 7.5 mins
+ several other non-conjugated PUFA's

A6.7. Calculations

Record peak height for 9,11-LA, internal standard. 9,12-LA.

For 9,11-LA - The internal standard [9,11-Linoleic acid (trans-trans)] also acts as a one-point external standard for 9,11-LA. The amount of internal standard in the assay (for serum) is 5μg: -

$$[9,11\text{-LA}] = \frac{\text{Peak height 9,11-LA}}{\text{Peak height Int. Std.}} \times 5 = A$$

:- Concentration in sample = $A \times \frac{1000}{0.5} \times \frac{1}{280.4}$ μM/L.

For 9,12-LA - Calculate the peak height ratio:

$$[9,12\text{-LA}] = \frac{\text{Peak height 9,12-LA}}{\text{Peak height Int. Std.}}$$ for all standards and samples

Plot standard curve of peak height ratio vs. Amount 9,12-LA in assay (μg).

Calculate amount 9,12-LA in 0.5ml sample from std. Curve = A

:- Concentration in sample = $A \times \frac{1000}{0.5} \times \frac{1}{280.4}$ μM/L.

A6.8. % Molar Ratio. The results are usually expressed as ratio 9,11-LA to 9,12-LA.

$$\frac{\text{Concentration 9,11-LA}}{\text{Concentration 9,12-LA}} \times 100\ \%$$

Typical values: 9,11-LA = 15 - 35 μM/L and 9,12-LA = 600 - 1200 μM/L.

Normal values (mean +/ - 2s.d.): % MR = 0.67 - 3.53.

A7.0. Urinary Inorganic Sulphate

A7.1. Principle

Turbidimetric technique based on the method of Lundquist 1980, in which inorganic sulphate in urine is determined spectrophotometrically as a barium sulphate suspension in polyethylene glycol 6000 solution.

A7.2. Reagents

Sodium Sulphate Nucleation Solution (50mM)

- Weigh 120 - 160mg $Na_2SO_4.10H_2O$ (MW = 322.19, BDH, AnalaR) into a 10ml screw-topped glass tube, to 1mg precision, recording the mass taken, "M" mg.
- Calculate volume "V", in ml, of deionised water required for 50mM solution, using the formula:-

  $V = 1000 \times M / (322.2 \times 50)$

  i.e. $V = M / 16.11$
- Add volume "V" of deionised water and vortex mix until dissolved (keeps indefinitely at room temperature).

Barium Chloride - Polyethylene Glycol 6000 Reagent (Ba-PEG Reagent)

- Dilute $BaCl_2.2H_2O$ (977mg, MW = 244.28, BDH AnalaR) and polyethylene glycol 6000 (15g, BDH) to 100ml with deionised water, with gentle warming to aid dissolution.
- Sonicate briefly to ensure complete dissolution, then transfer the solution to a 250ml beaker.
- Whilst stirring vigorously add dropwise 200ml Na_2SO_4 nucleation solution., store at 4°C, and replace weekly.

Potassium Sulphate Standard (10mM)

- Weigh 125 - 175mg K_2SO_4 (MW = 174.25, BDH, AnalaR) to 0.1mg precision in a 10ml screw-topped glass tube, recording the mass taken, "M" mg.
- Calculate volume "V", in ml, of deionised water required for 100mM solution, using the formula:-

$$V = 1000 \times M / (174.3 \times 100)$$

i.e. $V = M / 17.43$

- Add volume "V" of deionised water and vortex mix until dissolved.
- Dilute 1ml of this stock solution to 10ml with deionised water to produce 10mM working stock, (prepare freshly each day).

Hydrochloric Acid (0.5M)

- Dilute 4.9ml concentrated HCl (sp. gr. 1.16, 32%, BDH, AnalaR) to 100ml with deionised water, adding acid to water.
- Keeps indefinitely at room temperature.
-

A7.3. Equipment

Spectrophotometer (Cecil CE515), Vortex mixer and centrifuge.

A7.4. Procedure

- Assay 12 standards and up to 12 samples (in duplicate) in each run.

Standards

- Prepare inorganic sulphate standards as follows, from 10mM potassium sulphate solution and deionised water, in 10ml screw-capped tubes:

i-SO_4	**K_2SO_4**	**H_2O**
mmol	**ml**	**ml**
0	0	3000
0.25	25	2975
0.5	50	2950
0.75	75	2925
1	100	2900
1.25	125	2875
1.5	150	2850
2	200	2800
2.5	250	2750

3	300	2700
3.5	350	2650
4	400	2600

Samples

- Thaw batch of up to 12 samples, mix and centrifuge 5 min, 3,000 r.p.m. (2,000g).
- For each sample, pipette, in duplicate, 100ml urine then 2900ml deionised water into 10ml screw-capped tubes.

All Tubes

- Add 1ml HCl, and vortex mix.
- At 1 minute intervals, in batches of *ca.* 5 tubes, add 1ml Ba-PEG reagent and vortex mix.
- At precisely 5 min, measure absorbance at 600nm versus a water blank.

A7.5. Calculation

1mmol inorganic sulphate corresponds a concentration in urine of 10mM.

The standards should give an approximately linear variation of absorbance with K_2SO_4 content to 2.5mmoles (25mM) inorganic sulphate ($r > 0.998$, gradient $\approx$ 0.017 AU/mM). However, at the extremities of the standard range the graph deviates from linearity, so concentrations should be calculated from the curve rather than by linear regression.

Results were calculated using Microsoft Excel as follows:-

i) A standard curve is constructed from the absorbances for the 12 standards, and a cubic equation fitted.

ii) Inorganic sulphate concentration for mean sample absorbance is read from the curve.

A7.6. Quality Assurance

Accuracy -

If any calculated sample concentration exceeds 40mM, repeat the analysis in subsequent run, diluting the sample 1:1 with deionised water.

Precision -

Calculate difference (Δ) between duplicate values for sample pairs.

Acceptable precision: $\Delta \leq 0.02$

N.B. Excel will 'flag up' a warning if this limit is exceeded; analysis should then be repeated in subsequent run.

APPENDIX B Dietary information for challenge studies

Protein exchange - information sheets:

7G protein exchange:

1 oz lean, cooked meat / chicken
1 oz offal
¾ oz lean bacon
1½ oz white fish
2 fish fingers

1 hens egg (size 4)
1/3 pt milk
1 oz hard cheese
1¾ oz cottage cheese
1 carton yoghurt

5 oz baked beans
1 oz raw lentils
1 oz peanuts
4 oz fresh / frozen peas

2G protein exchange

1 large thin slice bread
1 oz pastry
½ oz raw pasta
1 oz raw rice

1 oz Corn Flakes
1 oz Rice Krispies
1 Weetabix

3 cream crackers
2 digestives

2 oz cream cheese
3 tsp. malted milk
1 oz milk chocolate

1 pkt crisps
1 oz Yorkshire Pudding

Typical meal pattern

Breakfast	
100ml fruit juice / fresh fruit	
Cereal e.g. 1 Weetabix / Shredded Wheat / 1 oz Corn Flakes / 1 oz Rice Krispies	2g
1 large thin slice bread / toast + margarine / butter + jam / marmalade.	2g
Tea / Coffee with milk from allowance.	
Lunch	
2 large thin slices bread + margarine / butter	4g
+ 1 oz meat / cheese / 1½ oz fish + / - salad as filling	7g
1 - 2 pieces fruit	
Evening meal	
2 oz meat / poultry / 3 oz fish	14g
5 oz boiled potato (or equivalent as roast / jacket / chips)	2g
vegetables	
During day	
½ pint milk	9g
~ 4 pieces fruit	2g
Suggested extras	
boiled sweets	
peppermints (up to 3 oz / day)	
fizzy drinks	

Meal patterns were written out in more detail than above, using exchange lists to keep meal patterns as close as possible to usual intake. Amounts of energy providing foods were also stipulated in line with caloric intake (Harris & Benedict equation). Along with a list of forbidden foods (depending upon the ability of the patient to organise their own diet within an exchange system).

APPENDIX C

Challenge study data sets

Plasma concentration profiles and AUC after oral loading with NAC and Methionine during challenge studies of :-

i) Taurine
ii) Serine
iii) Glycine
iv) Valine
v) Cystine
vi) Methionine
vii) GSH and
viii) Whole blood GSH.

Diagnoses of challenge study subject as reported in Appendix C

Healthy control	RAP	CP
HBS	*RMcE*	*JCN*
PFN	*EGY*	*JGH*
EGrY	*DHD*	*NHN*
MHN	*PHS*	*LJS*
AJN	*CKN*	*GMN*
GKY	*EMcE*	*POE*
NST	*JMK*	*EPY*
OSN	*SWS*	
	PFN	

NAC 1 - Oral NAC loading prior to AOT

NAC 2 - Oral NAC loading post AOT

METH 1 - Oral Methionine loading prior to AOT

METH 2 - Oral Methionine loading post AOT

	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
Initial	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**		**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	
HBS	55	60	61	73	54	60	72	53	559.8									
PFN	84	83	90	88	125	119		83	926.8									
EGrY	55	49	57	53	66	66	51	54	513.5									
MHN	109	104	156	107	92	53	107	77	838.0									
AJN	79	81	86	73	88	73	89	97	763.5									
GKY	61	82	72	73	74	105	109	71	785.5									
NST	59	94	90	73	62	140	140	97	943.3									
OSN	84	76	68	69	66	74	66	86	649.5									
mean	73.3	78.6	85.0	76.1	78.4	86.3	90.6	77.3	747.5									
sd	19.1	17.5	31.4	15.7	22.8	31.3	30.5	17.1	160.3									
RMcE	110		86	100	111	109	142	71	975.5	140	104	115	125	91	73	91	90	855.3
EGY	96	89	97	90	118	98	87	87	857.3	85	83	97	90	85	109	92	73	808.8
DHD	58	67	64	115	97	60	79	92	746.3	92	69	104	93	56	61	91	64	678.8
PHS	163	105	109	155	134	129	155	146	1265.0	118	78	86	159	161	101	110	74	1022.0
CKN	84	40	37	71	67	57	42	35	463.5	53	122	137	52	37	56	76	60	595.3
EMcE	97	80	86	84	70	78	79	129	784.5	118	120	106		105		93	106	922.5
JMK	103	113	142	123	113	104	142	110	1086.3	85	71	96	88	90	93	89	79	786.0
SWS	101	83	67	114	112	97	76	135	901.3	90	99	103	102	120	89	99	106	920.8
PFH	92	73	73	79	74	91	82	107	767.5	not	rechall enged		with drew					
mean	100.4	81.3	84.6	103.4	99.6	91.4	98.2	101.3	871.9	97.6	93.3	105.5	101.3	93.1	83.1	92.6	81.5	823.7
sd	27.8	22.7	30.0	26.2	23.9	23.2	38.5	34.7	227.3	26.8	21.1	15.3	33.4	37.9	20.2	9.5	17.6	138.5
JCN	121	107	110	119	81	114	108	111	959.8	114	115	76	74	98	85	114	143	943.5
JGH	112	156	109	119	122	116	85	115	1000.3	93	115	127	143	119	135	122	114	1113.8
NHN	67	83	110	97	93	107	80	94	831.3	not	rechall enged		with drew					
LJS	122	70	74	116	98	112	103	135	967.8	130	157	129	143	104	90	140	76	1038.5
GMN	70	58	58	78	69	57	110	91	726.5	84	91	117	98	73	94	110	86	850.0
POE	95	92	107	104	102	90	102	95	887.3	134	131	126	158	86	90	110	77	947.0
EPY	43	68	95	61	80	65	73	79	653.5	68	74	97	79	60	73	113	72	743.3
mean	90.0	90.6	94.7	99.1	92.1	94.4	94.4	102.9	860.9	103.8	113.8	112.0	115.8	90.0	94.5	118.2	94.7	939.3
sd	30.6	33.2	20.8	22.4	17.5	24.5	14.8	18.7	131.2	26.4	29.2	21.2	36.5	21.5	21.1	11.6	28.1	131.7

Appendix C **Plasma Taurine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
Initial	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**		**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	
HBS	56	79	60	104	50	43	56	154	684.0									
PFN	94	68	106	79	66	89	87	93	757.3									
EGrY	64	46	35	42	28	39	42	44	359.5									
MHN	94	80	70	93	83	65	74	85	707.5									
AJN	101	68	87	72	113	94	115	116	918.0									
GKY	65	81	61	93	102	68	89	62	728.5									
NST	89	63	77	71	87	108	121	84	854.0									
OSN	76	123	97	78	75	106	84	77	783.0									
mean	79.9	76.0	74.1	79.0	75.5	76.5	83.5	89.4	724.0									
sd	16.8	22.2	22.8	18.9	27.5	26.9	26.8	33.6	166.4									
RMcE	78	89	113		144	159	102	148	1147.3	77	92	124	77	115	86	89	97	851.5
EGY	92	98	101	71	87	83	72	77	726.0	92	77	62	75	72	80	83	74	693.3
DHD	138	112	125	84	115	76	119	93	930.8	100	114	105	92	112	91	141	104	1002.2
PHS	149	149	109	126	149	106	104	153	1138.3	96	85	77	80	104	102	99	93	856.3
CKN	58	87	84	59	66	58	54	75	579.0	70	69	56	72	61	56	82	89	645.5
EMcE	86	70	61	60	61	86	49	59	566.3	110	108	100	116	106	128	159	112	1124.3
JMK		126	109	125	101	108	161	157		140	82	89	120	59	97	78	140	860.0
SWS	111	100	142	80	92	96	99	98	880.5	87	83	102	155	128	78	120	143	1062.8
PFH	77	70	69	77		92	79	89	743.3	not	rechall enged		with drew					
mean	98.6	100.1	101.4	85.3	101.9	96.0	93.2	105.4	838.9	96.5	88.8	89.4	98.3	94.6	89.7	106.4	106.4	887.0
sd	31.6	25.8	26.1	26.4	32.6	28.2	34.7	37.3	226.3	21.6	15.3	23.1	29.4	26.6	20.9	30.4	24.3	168.7
JCN	72	176	74	161	94	74	101	132	981.3	133	193	195	176	133	182	203	163	1577.0
JGH	98	108	134	92	122	112	142	175	1170.5	106	102	117	95	69	81	97	95	816.8
NHN	48	71	68		76	119	102	150	898.5	not	rechall enged		with drew					
LJS		79	83	55	54	87	97	127		63	68	68	45	60	92	86	103	704.8
GMN	72	69	89	92	77	89	105	118	851.3	82	125	65	113	88	110	86	107	879.5
POE		121	110	120	113	134	102	111		110	92	96	73	82	85	70	70	707.5
EPY	107	100	111	81	94	69	93	61	758.5	88	104	116	113	130	139	101	101	1027.3
mean	79.4	103.4	95.6	100.2	90.0	97.7	106.0	124.9	932.0	97.0	114.0	109.5	102.5	93.7	114.8	107.2	106.5	952.1
sd	23.5	37.5	23.6	36.4	23.3	24.3	16.4	35.4	155.8	24.5	42.9	47.5	44.4	30.9	39.2	48.2	30.7	328.9

Appendix C **Plasma Taurine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
Initial	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**		**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	
HBS	101	107	105	99	96	97	97	76	852.0									
PFN	95	121	123	107	88	97		134	977.3									
EGrY	176	186	230	215	210	185	147	191	1676.8									
MHN	104	135	114	106	99	107	101	82	915.8									
AJN	107	139	101	104	94	87	74	99	837.3									
GKY	78	91	85	83	80	80	110	78	795.0									
NST	119	137	131	130	120	109	138	111	1114.3									
OSN	107	123	117	104	110	87	88	126	933.3									
mean	110.9	129.9	125.8	118.5	112.1	106.1	107.9	112.1	1,012.7									
sd	28.8	28.0	44.4	41.0	41.4	33.4	26.3	38.6	285.9									
RMcE	58		83	77	58	62	69	54	584.5	89	86	81	79	79	70	89	73	718.0
EGY	137	128	152	134	104	110	100	110	1019.3	106	91	111	108	98	84	92	88	847.5
DHD	146	145	129	130	110	108	94	135	1044.5	121	125	122	140	99	109	139	111	1085.0
PHS	109	132	137	122	83	95	125	89	965.5	136	158	155	150	144	99	128	107	1153.5
CKN	99	91	56	84	96	90	74	93	767.3	103	98	88	92	84		109	100	876.8
EMcE	97	105	98	91	83	83	107	105	864.0	99	98	91		72		88	79	750.0
JMK	45	56	65	55	42	103	129	121	816.0	115	138	139	130	103	87	96	93	937.8
SWS	122	133	128	128	105	106	110	117	1028.5	159	164	167	143	125	142	128	127	1227.3
PFH	99	106	101	94	87	82	91	97	826.0	not	rechall	enged		with	drew			
mean	101.3	112.0	105.4	101.7	85.3	93.2	99.9	102.3	879.5	116.0	119.8	119.3	120.3	100.5	98.5	108.6	97.3	949.5
sd	33.2	28.9	33.3	27.9	22.6	15.7	20.5	23.3	151.3	22.6	30.9	32.2	27.5	24.1	25.1	20.4	17.7	187.6
JCN	93	118	122	123	101	83	108	106	944.3	57	80	69	45	50	64	54	59	514.8
JGH	84	95	90	89	61	61	73	96	693.8	102	105	112	109	70	72	88	98	801.0
NHN	107	138	148	146	141	121	116	106	1128.8	not	rechall	enged		with	drew			
LJS	94	89	93	85	77	90	97	89	801.8	128	145	146	143	130	106	136	116	1154.5
GMN	54	53	57	76	48	51	66	83	566.0	66	73	76	73	53	48	57	62	536.8
POE	114	109	113	128	106	91	86	97	902.0	113	87	114	116	97	98	105	105	931.0
EPY	111	156	141	128	107	113	131	104	1085.0	133	154	166	146	138	126	123	123	1196.5
mean	93.9	108.3	109.1	110.7	91.6	87.1	96.7	97.3	874.5	99.8	107.3	113.8	105.3	89.7	85.7	93.8	93.8	855.8
sd	20.7	33.8	31.8	26.9	31.7	25.3	23.4	8.9	203.2	31.8	34.5	37.9	39.7	38.3	29.2	33.9	27.2	294.0

Appendix C **Plasma Serine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

	METH1									METH2								
	Time mins								AUC	*Time mins*								AUC
Initial	**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**		**0**	**30**	**60**	**90**	**180**	**270**	**360**	**540**	
HBS	113	126	120	115	112	105	116	105	1010.3									
PFN	108	113	121	109	89	83	92	143	932.5									
EGrY	128	135	100	131	115	109	115	125	1062.8									
MHN	118	130	128	120	126	112	125	109	1080.3									
AJN	106	85	117	93	94	83	71	75	758.3									
GKY	57x	77	72	79	73	70	74	75										
NST	104	105	103	118	94	93	90	92	869.0									
OSN	123	125	114	100	98	91	108	107	937.3									
mean	114.3	112.0	109.4	108.1	100.1	93.3	98.9	103.9	950.0									
sd	9.1	21.5	17.7	16.7	16.8	14.6	20.1	23.4	113.4									
RMcE	80	75	72		58	46	52	50	510.0	86	95	79	77	72	75	75	71	681.3
EGY	122	118	118	119	97	88	103	101	928.3	108	108	112	107	106	88	94	117	922.0
DHD	120	108	107	101	97	87	120	112	952.5	130	134	132	128	110	104	122	101	1038.6
PHS	124	109	117	101	93		112	104	946.3	109	120	125	95	93	82	134	121	990.3
CKN	64	86	78	67	87	73	71	91	701.3	117	122	138	132	129	89	103	110	1015.0
EMcE	99	101	91	91	87	89	87	97	817.0	89	92	85	89	74	83	84	78	741.3
JMK	102	87	121	97	91	123	118	132	1011.0	64	68	75	72	63	72	116	107	783.5
SWS	139	142	125	115	107	99	112	106	1003.3	109	107	108	96	94	93	105	100	897.5
PFH	94	96	93	89		77	84	107	796.5	not	rechall enged		with drew					
mean	104.9	102.4	102.4	97.5	89.6	85.3	95.4	100.0	851.8	101.5	105.7	106.8	99.4	92.7	85.8	104.1	100.6	883.7
sd	23.7	20.0	19.6	16.2	14.3	22.0	23.5	22.0	164.7	20.7	20.6	24.6	21.7	22.2	10.3	19.6	17.7	133.9
JCN	87	98	99	84	89	78	84	70	748.8	67	84	78	79	88	88	87	80	756.5
JGH	77	81	83	77	88	67	115	94	810.5	100	92	97	94	75	85	108	99	845.0
NHN	87	87	92	103	109	101	97	98	894.5	not	rechall enged		with drew					
LJS		97	113	110	99	105	112	106		118	111	114	96	104	99	132	120	1019.5
GMN	63	65	66	72	56	50	65	79	577.0	66	64	49	62	47	45	58	58	490.5
POE	139	150	120	128	119	115	123	108	1087.5	92	99	84	94	95	85	95	95	834.8
EPY	102	114	124	119	117	91	150	116	1087.0	124	126	133	124	121	119	120	127	1105.0
mean	92.5	98.9	99.6	99.0	96.7	86.7	106.6	95.9	867.5	94.5	96.0	92.5	91.5	88.3	86.8	100.0	96.5	841.9
sd	26.2	27.2	21.0	21.7	21.8	23.0	27.6	16.4	199.5	24.6	21.5	29.3	20.6	25.5	24.3	26.3	25.5	215.2

Appendix C **Plasma Serine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
Initial	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	190	185	183	174	188	183	179	152	1592.8									
PFN	291	342	328	295	304	283		312	2709.8									
EGrY	510	463	542	532	562	480	449	493	4474.8									
MHN	170	180	161	159	158	167	140	150	1399.5									
AJN	210	235	171	180	177	174	157	206	1624.3									
GKY	225	260	236	252	254	282	270	235	2320.3									
NST	273	275	267	291	298	293	407	304	2888.5									
OSN	356	370	351	336	341	352	315	300	2983.8									
mean	278.1	288.8	279.9	277.4	285.3	276.8	273.9	269.0	2,499.2									
sd	111.4	97.3	127.4	121.7	130.1	106.0	122.9	111.6	1,011.5									
RMcE	130		126	130	126	161	178	132	1318.5	167	160	139	130	132	129	151	139	1261.0
EGY	441	420	369	409	394	417	407	377	3611.5	349	350	357	331	351	347	358	339	3132.8
DHD	392	348	310	287	282	268	246	320	2572.5	252	252	248	266	212	258	288	224	2268.0
PHS	196	214	216	196	173	171	213	179	1723.8	249	258	238	227	250	178	229	192	1982.5
CKN	152	117	114	117	116	124	128	150	1143.5	273	246	233	241	238		315	293	2468.8
EMcE	198	172	156	148	166	186	204	185	1626.0	203	198	188		129		176	169	1488.8
JMK	149	159	147	145	132	207	253	216	1737.0	225	199	166	182	158	162	170	169	1536.8
SWS	275	287	270	285	268	231	250	301	2394.8	366	356	342	299	296	295	275	284	2670.8
PFH	217	217	211	207	213	203	203	235	1908.5	not	rechall enged		with drew					
mean	238.9	241.8	213.2	213.8	207.8	218.7	231.3	232.8	2004.0	260.5	252.4	238.9	239.4	220.8	228.2	245.3	226.1	2101.2
sd	110.2	102.7	88.0	96.4	92.0	85.1	76.9	83.3	756.0	68.3	70.4	78.0	68.4	79.4	85.1	75.5	71.6	650.8
JCN	213	224	200	199	179	167	187	195	1696.5	111	129	121	82	103	103	108	114	957.8
JGH	249	292	269	190	182	182	166	212	1770.3	258	234	221	209	234	228	270	241	2163.0
NHN	201	239	242	248	247	231	225	212	2080.0	not	rechall enged		with drew					
LJS	199	160	168	162	166		142	178	1442.3	230	226	209	180	198	156	191	171	1672.3
GMN	186	164	186	207	175	196	238	247	1891.0	213	204	212	232	193	184	202	256	1897.3
POE	241	215	221	209	207	175	182	192	1757.8	165	128	155	164	145	159	177	185	1478.5
EPY	340	367	355	361	359	359	374	349	3249.0	317	342	369	331	333	290	307	322	2874.0
mean	232.7	237.3	234.4	225.1	216.4	218.3	216.3	226.4	1983.8	215.7	210.5	214.5	199.7	201.0	186.7	209.2	214.8	1840.5
sd	52.5	72.8	63.1	65.2	68.6	72.5	76.9	58.3	590.4	71.8	79.5	85.1	82.3	79.2	65.0	70.6	73.3	650.2

Appendix C **Plasma Glycine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

Initial	METH1 Time mins 0	30	60	90	180	270	360	540	AUC	METH2 Time mins 0	30	60	90	180	270	360	540	AUC
HBS	213	198	198	203	199	183	191	196	1751.0									
PFN	305	291	279	255	268	248	269	374	2556.5									
EGrY	337	306	220	274	246	249	245	259	2303.5									
MHN	184	174	187	175	188	148	166	160	1519.0									
AJN	198	146	210	165	182	152	140	167	1459.0									
GKY	162x	217	200	219	213	210	208	206										
NST	213	207	198	217	221	236	272	209	2083.8									
OSN	288	269	241	208	230	207	206	254	2035.0									
mean	248.3	226.0	216.6	214.5	218.4	204.1	212.1	228.1	1,958.3									
sd	60.3	57.1	30.2	36.6	29.3	40.2	47.4	68.8	404.9									
RMcE	181	183	173		147	143	154	127	1361.8	144	154	121	118	160	173	187	168	1463.8
EGY	380	407	377	343	317	332	308	326	2985.5	369	378	355	357	355	325	324	305	3022.3
DHD	297	246	234	233	225	212	242	242	2110.3	295	292	270	266	247	234	291	238	2353.3
PHS	219	207	209	186	181		219	196	1807.0	213	228	219	182	192	175	282	244	2009.8
CKN	176	165	154	141	146	139	140	156	1321.0	243	246	230	241	237	275	306	270	2401.3
EMcE	184	171	158	151	150	143	145	137	1332.8	156	155	137	138	130	137	143	123	1229.8
JMK	205	192	182	204	214	213	226	202	1894.3	146	146	133	157	131	163	147	139	1313.3
SWS	296	293	256	240	240	235	254	227	2213.0	280	260	271	257	235	234	245	212	2165.3
PFH	236	212	209	196		188	194	214	1793.0	not	rechall enged		with drew					
mean	241.6	230.7	216.9	211.8	202.5	200.6	209.1	203.0	1868.7	230.7	232.3	217.0	214.5	210.8	214.5	240.6	212.3	1994.8
sd	69.2	77.3	69.1	63.4	59.3	64.7	56.4	61.0	533.7	81.4	80.4	82.5	80.2	74.8	63.9	72.4	64.2	622.3
JCN	191	197	205	159	158	155	185	170	1548.5	160	132	164	164	163	157	160	158	1429.0
JGH	221	207	205	225	213	213	245	262	2069.5	258	226	250	228	194	216	273	243	2124.3
NHN	185	180	189	227	227	212	205	222	1910.5	not	rechall enged		with drew					
LJS		297	297	246	238	205	193	195		235	222	187	152	161	147	170	159	1498.3
GMN	239	215	208	214	212	189	210	218	1886.3	251	229	157	210	171	195	207	242	1843.5
POE	212	198	185	189	194	182	211	204	1778.3	168	163	148	145	133	129	139	153	1277.8
EPY	389	365	348	316	334	323	375	326	3088.0	323	317	301	282	253	258	278	293	2503.3
mean	239.5	237.0	233.9	225.1	225.1	211.3	232.0	228.1	2046.8	232.5	214.8	201.2	196.8	179.2	183.7	204.5	208.0	1779.3
sd	75.9	68.0	62.9	49.2	54.5	53.3	65.8	51.5	538.4	61.0	63.8	61.2	53.2	41.1	48.5	59.3	59.2	469.1

Appendix C **Plasma Glycine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
Initial	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	234	225	206	187	182	218	225	184	1843.3									
PFN	166	176	156	130	125	138		214	1420.5									
EGrY	196	168	175	161	179	167	144	188	1506.5									
MHN	278	278	241	220	212	264	268	216	2190.0									
AJN	291	313	220	209	187	182	157	253	1834.5									
GKY	186	206	180	176	170	211	282	230	1966.5									
NST	247	239	210	217	216	204	241	251	2052.0									
OSN	277	315	287	266	262	263	255	347	2518.0									
mean	234.4	240.0	209.4	195.8	191.6	205.9	224.6	235.4	1,916.4									
sd	47.2	57.4	41.5	41.7	39.9	43.9	53.9	51.8	355.3									
RMcE	220		261	250	210	228	255	227	2127.0	348	321	287	274	238	233	261	254	2339.8
EGY	280	260	266	222	190	212	227	296	2112.8	203	210	202	186	175	165	165	191	1610.5
DHD	262	242	210	202	182	188	180	274	1864.5	233	226	208	228	155	186	249	239	1933.5
PHS	210	207	195	170	144	159	251	171	1699.3	188	186	167	156	194	164	229	210	1746.8
CKN	250	216	201	197	170	160	163	222	1662.8	277	258	234	230	204		292	281	2301.8
EMcE	284	263	241	229	215	226	307	282	2327.3	266	261	233		153		290	254	2121.8
JMK	194	207	205	182	167	273	268	207	2010.0	195	194	184	185	137	145	157	196	1493.0
SWS	259	252	222	217	196	193	209	274	1983.5	307	296	278	250	218	231	231	309	2270.5
PFH	210	206	192	179	173	174	178	205	1659.0	not	rechall enged		with drew					
mean	241.0	231.6	221.4	205.3	183.0	201.4	226.4	239.8	1938.4	252.1	244.0	224.1	215.6	184.3	187.3	234.3	241.8	1977.2
sd	33.1	25.1	28.1	26.3	22.4	37.2	48.0	43.0	234.8	57.5	48.5	42.5	41.5	35.2	37.0	51.0	41.5	331.2
JCN	199	223	189	188	150	122	168	167	1480.3	162	159	161	121	140	123	125	128	1189.3
JGH	202	216	203	183	157	140	147	187	1499.8	219	209	209	162	178	155	161	177	1553.0
NHN	181	214	209	205	213	219	227	209	1934.0	not	rechall enged		with drew					
LJS	149	161	137	128	131	163	197	139	1407.0	176	146	145	128	151	147	160	125	1312.0
GMN	128	121	121	132	99	106	148	165	1173.0	153	146	145	143	114	106	131	145	1169.0
POE	254	228	223	218	183	192	182	229	1822.5	233	173	213	204	175	199	227	218	1854.0
EPY	172	198	172	167	156	143	172	169	1484.0	213	213	203	180	202	209	210	231	1876.8
mean	183.6	194.4	179.1	174.4	155.6	155.0	177.3	180.7	1542.9	192.7	174.3	179.3	156.3	160.0	156.5	169.0	170.7	1492.3
sd	40.7	39.4	38.1	34.4	36.3	39.6	28.2	30.2	256.8	33.2	30.1	32.5	31.9	31.3	40.8	41.4	45.8	319.7

Appendix C **Plasma Valine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

Initial	METH1								AUC	METH2								AUC
	Time mins									*Time mins*								
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	274	280	270	255	252	230	239	259	2247.8									
PFN	165	159	157	129	111	104	133	187	1230.5									
EGrY	165	155	117	148	122	115	141	151	1224.5									
MHN	273	269	271	255	257	226	259	215	2223.0									
AJN	287	216	290	229	210	173	194	200	1864.8									
GKY		199	188	207	179	190	210	204										
NST	293	285	280	298	215	208	218	241	2140.3									
OSN	299	306	291	265	260	240	311	303	2542.5									
mean	250.9	233.6	233.0	223.3	200.8	185.8	213.1	220.0	1,924.8									
sd	59.4	59.2	68.5	58.8	58.9	51.9	59.0	46.9	516.0									
RMcE	318	298	284		226	190	218	240	2114.5	312	339	277	259	234	237	231	243	2235.8
EGY	275	285	272	260	209	200	200	209	1984.3	198	221	214	227	210	187	188	275	1925.0
DHD	274	242	239	227	204	200	240	297	2127.5	275	275	262	237	192	182	200	216	1908.0
PHS	176	171	175	160	125		179	193	1484.8	184	198	188	162	144	109	186	201	1500.5
CKN	327	323	311	292	288	256	248	293	2504.3	235	221	249	245	228	188	209	223	1967.5
EMcE	256	249	242	227	213	243	270	307	2288.5	266	266	236	237	200	217	244	234	2080.0
JMK	200	201	193	204	205	254	250	294	2143.0	163	169	150	181	118	148	166	168	1405.8
SWS	318	284	284	260	224	223	241	243	2200.8	265	250	256	240	210	209	234	248	2086.3
PFH	227	224	221	207		155	152	273	1741.8	not	rechall	enged	with	drew				
mean	263.4	253.0	246.8	229.6	211.8	215.1	222.0	261.0	2065.5	237.2	242.3	229.0	223.5	192.0	184.7	207.2	226.0	1888.6
sd	54.0	49.1	45.2	41.0	44.4	35.3	38.2	41.5	300.3	51.4	52.4	42.6	33.7	40.6	40.5	27.3	32.4	289.8
JCN	212	207	220	188	152	132	186	149	1522.5	244	219	250	256	261	253	279	276	2364.3
JGH	168	160	175	170	117	99	141	166	1269.8	219	204	226	193	163	167	195	179	1665.0
NHN	146	145	146	170	164	160	167	173	1473.3	not	rechall	enged	with	drew				
LJS		169	165	148	136	143	178	162		148	159	132	117	133	129	194	184	1405.0
GMN	159	166	163	173	124	108	161	263	1482.0	203	196	131	179	128	126	151	178	1381.0
POE	185	145	162	165	161	152	174	185	1503.3	196	219	183	190	166	145	167	202	1585.3
EPY	232	214	204	198	174	158	225	247	1839.8	178	174	176	166	154	154	167	184	1499.3
mean	183.7	172.3	176.4	173.1	146.9	136.0	176.0	192.1	1515.1	198.0	195.2	183.0	183.5	167.5	162.3	192.2	200.5	1650.0
sd	32.9	27.8	26.2	16.1	21.6	24.3	25.9	44.5	183.6	33.1	24.4	48.4	45.0	48.4	47.0	45.9	38.0	366.0

Appendix C **Plasma Valine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

Initial	NAC1								AUC	NAC2								AUC
	Time mins									*Time mins*								
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	102	152	130	134	103	86	76	65	852.5									
PFN	127	243	264	217	159	140		127	1446.5									
EGrY	105	161	159	160	179	129	99	105	1188.5									
MHN	140	188	174	161	125	124	100	105	1133.0									
AJN	121	185	155	123	92	95	78	93	918.8									
GKY	122	180	188	178	174	167	144	112	1396.0									
NST	117	169	159	152	131	107	105	99	1087.0									
OSN	105	124	117	109	85	75	66	80	764.3									
mean	117.4	175.3	168.3	154.3	131.0	115.4	95.4	98.3	1,098.3									
sd	13.0	34.4	44.8	33.8	36.6	30.5	25.9	19.2	246.0									
RMcE	94		167	137	126	104	104	84	1014.3	150	170	206	187	140	112	108	112	1201.5
EGY	141	129	102	105	96	111	108	109	972.8	127	131	117	90	90	92	90	101	872.8
DHD	153	215	209	247	194	157	126	125	1494.8	127	152	159	215	125	112	125	87	1169.5
PHS	112	187	209	202	135	99	108	94	1163.0	118	169	189	230	221	121	120	96	1365.5
CKN	102	137	144	149	114	84	70	83	894.0	101	137	121	125	111		99	98	973.0
EMcE	132	169	182	186	127	105	109	106	1146.8	170	167	154		120		106	111	1103.0
JMK	93	158	224	195	164	114	110	85	1201.3	116	160	144	123	109	96	103	109	1006.8
SWS	140	199	209	185	156	129	114	125	1295.5	170	232	259	258	185	157	145	138	1592.3
PFH	140	205	238	248	207	177	149	130	1610.8	not	rechall enged		with drew					
mean	123.0	174.9	187.1	183.8	146.6	120.0	110.9	104.6	1199.2	134.9	164.8	168.6	175.4	137.6	115.0	112.0	106.5	1160.5
sd	22.9	31.9	43.2	47.7	36.8	29.7	20.7	19.0	236.8	25.7	30.9	47.5	63.4	43.9	23.3	17.4	15.3	231.5
JCN	132	231	219	230	199	141	130	120	1470.5	114	126	144	87	88	77	73	93	801.8
JGH	112	114	156	163	142	110	95	103	1072.3	156	179	208	192	143	125	119	117	1269.8
NHN	96	155	179	171	127	111	100	92	1082.0	not	rechall enged		with drew					
LJS	80	180	176	154	138	105	84	70	1010.5	106	157	165	148	144	94	93	78	1018.8
GMN	97	138	182	216	176	150	153	128	1425.5	148	217	209	217	172	141	128	127	1415.0
POE	142	139	170	187	172	129	103	106	1219.3		no	results	avail able					
EPY	111	222	268	241		143	128	101	1455.8	122	220	249	240	178	143	145	127	1503.3
mean	110.0	168.4	192.9	194.6	159.0	127.0	113.3	102.9	1248.0	129.2	179.8	195.0	176.8	145.0	116.0	111.6	108.4	1201.7
sd	21.5	44.4	38.3	34.5	27.6	18.3	24.3	18.9	199.9	21.8	40.0	41.2	60.7	35.6	29.3	28.6	21.9	289.2

Appendix C **Plasma Cystine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

Initial	METH1 Time mins 0	30	60	90	180	270	360	540	AUC	METH2 Time mins 0	30	60	90	180	270	360	540	AUC
HBS	120	136	146	133	134	117	124	124	1145.5									
PFN	140	160	158	156	149	138	127	145	1283.8									
EGrY	79	89	67	117	101	102	87	78	832.0									
MHN	166	179	195	174	182	171	174	176	1587.5									
AJN	114	94	124	102	103	93	93	94	883.8									
GKY	94	129	130	145	126	125	129	129	1158.3									
NST	104	104	115	111	98	96	125	84	944.8									
OSN	106	115	115	105	113	105	94	96	929.0									
mean	115.4	125.8	131.3	130.4	125.8	118.4	119.1	115.8	1,095.6									
sd	27.2	31.8	37.2	26.1	28.9	26.2	28.2	33.9	253.2									
RMcE	146	128	130		118	99	102	99	996.0	143	162	138	126	119	124	113	120	1110.5
EGY		no	results	avail able						129	145	143	154	156	147	129	126	1264.0
DHD	147	142	155	153	154	146	154	138	1341.8		no	results	avail able					
PHS	123	114	129	129	113		118	109	1053.0	112	122	128	114	126	113	115	107	1044.8
CKN	113	114	115	116	109	92	101	104	943.5	90	99	102	93	112			96	924.0
EMcE	126	126	126	121	112	131	129	111	1099.8	115	119	109	117	102	102	105	104	958.0
JMK	101	113	134	140	127	135	137	131	1186.5	105	105	102	105	87	93	99	101	879.0
SWS	148	141	151	143	149	149	154	152	1347.5	141	135	135	134	132	133	139		
PFH										not	rechall enged		with drew					
mean	129.1	125.4	134.3	133.7	126.0	125.3	127.9	120.6	1138.3	119.3	126.7	122.4	120.4	119.1	118.7	116.7	109.0	1030.0
sd	18.5	12.5	14.1	14.1	18.4	24.2	22.1	19.9	160.4	19.4	22.2	17.7	19.9	22.2	20.0	14.9	11.6	141.9
JCN		124	133	133	156	145	151	129		120	128	138	155	160	170	199	178	1527.8
JGH	129	130	146	134	143	154	141	139	1275.5	128	127	142	140	127	122	126	125	1151.0
NHN	86	82	84	98	102	92	88	93	831.0	not	rechall enged		with drew					
LJS		87	90	84	98	102	111	110		90	89	95	84	84	78	89	85	769.3
GMN	148	151	150	167	183	203	233	229	1801.3					154		153		
POE	138	133	137	144	128	118	114	117	1114.5	115	125	114	113	120	112	112	121	1042.8
EPY	152	136	152	153	149	119	122	113	1181.0	125	119	131	140	131	122	119	127	1134.0
mean	130.6	120.4	127.4	130.4	137.0	133.3	137.1	132.9	1240.7	115.6	117.6	124.0	126.4	129.3	120.8	133.0	127.2	1125.0
sd	26.5	25.9	28.5	29.6	30.2	37.7	47.0	44.8	354.5	15.1	16.4	19.4	28.1	27.2	32.9	38.4	33.2	272.2

Appendix C **Plasma Cystine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

Initial	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	25	22	21	18	18	23	22	19	185.3									
PFN	21	21	19	17	15	20		32	196.8									
EGrY	25	20	23	21	24	22	21	22	198.0									
MHN	21	21	17	14	14	19	22	14	158.3									
AJN	28	25	19	18	16	16	16	30	176.0									
GKY	23	25	22	20	19	23	39	24	236.0									
NST	31	29	25	26	26	21	28	28	236.3									
OSN	18	23	19	17	17	17	19	29	179.8									
mean	24.0	23.3	20.6	18.9	18.6	20.1	23.9	24.8	195.8									
sd	4.2	3.0	2.6	3.6	4.3	2.6	7.6	6.2	27.8									
RMcE	20		22	19	17	16	22	21	176.0	34	30	24	20	18	17	22	19	186.0
EGY	23	22	18	19	14	16	18	20	160.3	21	17	15	16	15	17	15	23	153.5
DHD	27	22	25	16	20	14	15	33	180.5	23	20	20	21	12	16	27	23	184.0
PHS	22	20	18	17	14	17	32	18	187.0	24	24	23	20	24	20	30	26	222.0
CKN	22	14	13	12	10	12	13	18	120.3	27	19	16	15	19	14	31	25	196.0
EMcE	19	18	14	13	13	14	23	20	156.0	19	20	17				26	17	191.0
JMK	26	28	24	23	21	43	34	40	288.0	28	26	21	21	17	14	14	16	153.5
SWS	20	22	18	18	17	17	20	28	181.0	28	26	22	18	15	16	19	32	186.3
PFH	21	18	17	16	14	14	16	20	146.8	not	rechall enged		with drew					
mean	22.2	20.5	18.8	17.0	15.6	18.1	21.4	24.2	177.3	25.5	22.8	19.8	18.7	17.1	16.3	23.0	22.6	184.0
sd	2.7	4.1	4.1	3.3	3.5	9.5	7.3	7.8	46.5	4.8	4.4	3.4	2.4	3.8	2.1	6.5	5.3	22.4
JCN	21	24	18	18	15	10	18	15	144.8	12	17	14	9	10	11	14	12	108.5
JGH	19	22	20	15	14	10	16	22	145.8	23	23	27	17	17	17	19	21	173.0
NHN	23	26	23	24	25	27	27	23	227.5	not	rechall enged		with drew					
LJS	18	15	16	12	12	15	25	15	151.3	17	10	14	13	15	13	15	12	123.0
GMN	14	11	16	22	9	10	19	24	146.3	23	21	16	19	11	11	13	20	135.5
POE	23	20	17	18	21	16	21	20	175.0	29	18	22	23	17	18	24	23	191.3
EPY	22		43	17	17		26	28	218.5	35	37	29	23	27	25	20	23	222.3
mean	20.0	19.7	21.9	18.0	16.1	14.7	21.7	21.0	177.4	23.2	21.0	20.3	17.3	16.2	15.8	17.5	18.5	158.9
sd	3.3	5.7	9.7	4.0	5.4	6.6	4.3	4.8	36.0	8.2	9.0	6.7	5.6	6.1	5.4	4.2	5.2	43.9

Appendix C **Plasma Methionine (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

Initial	METH1 Time mins 0	30	60	90	180	270	360	540	AUC	METH2 Time mins 0	30	60	90	180	270	360	540	AUC
HBS	26	305	319	309	395	336	274	117	2516.0									
PFN	27	114	310	297	321	187	125	72	1667.0									
EGrY	16	140	149	184	180	97	64	34	943.0									
MHN	23	169	186	204	480	410	344	271	2902.8									
AJN	31	99	232	237	266	231	197	148	1821.0									
GKY	18	127	192	248	257	254	207	121	1825.8									
NST	23	164	166	293	233	240	233	102	1850.5									
OSN	25	222	319	320	317	299	211	84	2121.5									
mean	23.6	167.5	234.1	261.5	306.1	256.8	206.9	118.6	1,955.9									
sd	4.8	67.4	71.9	50.7	95.4	94.8	85.5	70.6	584.7									
RMcE	29	181	250		256	238	209	159	2177.0	32	97	232	388	353	375	313	250	2731.8
EGY	21	68	158	246	311	232	147	71	1616.0	17	184	213	334	306	211	139	68	1727.0
DHD	19	191	258	254	337	226	160	78	1804.8	22	195	260	320	330	222	158	67	1835.4
PHS	24	113	210	219	201	145	118	62	1264.0	24	139	169	178	125	79	73	48	880.3
CKN	26	213	159	220	330	291	207	105	1967.3	19	88	129	121	373	241	182	91	1701.3
EMcE	17	112	197	302	354	327	281	186	2393.5	17	152	234	327	375	344	300	192	2565.8
JMK	24	165	365	398	403	345	289	183	2715.8	22	48	258	299	322	327	259	164	2259.8
SWS	27	43	311	418	302	236	188	78	1948.8	26	161	166	167	129	97	71	39	894.3
PFH	21	221	238	287		290	236	143	2135.0	not	rechall	enged	with	drew				
mean	23.1	145.2	238.4	293.0	311.8	258.9	203.9	118.3	1929.1	22.3	133.0	207.6	266.8	289.1	237.0	186.8	114.8	1824.4
sd	3.9	63.9	68.1	76.8	61.6	61.0	58.1	49.8	425.4	5.0	51.0	47.7	97.0	102.9	109.6	95.2	77.4	691.1
JCN	26	61	163	171	230	178	131	60	1286.3	21	43	89	133	144	164	110	51	990.3
JGH	12	88	227	266	224	196	157	96	1553.8	24	55	234	247	207	169	133	63	1355.3
NHN	18	80	150	235	309	248	200	133	1839.5	not	rechall	enged	with	drew				
LJS	22	184	274	250	382	362	297	205	2576.3	22	88	245	269	321	287	246	140	2116.5
GMN	20	27	42	172	190	179	136	82	1194.0	23	76	107	272	195	167	104	47	1216.8
POE	26	69	151	283	438	378	303	188	2587.3	27	154	184	242	279	247	224	135	1913.3
EPY	29	121	120	182	170	97	93	52	997.5	25	114	178	149	155	126	102	69	1055.8
mean	21.9	90.0	161.0	222.7	277.6	234.0	188.1	116.6	1719.2	23.7	88.3	172.8	218.7	216.8	193.3	153.2	84.2	1441.3
sd	5.8	50.3	74.4	47.1	101.7	103.0	82.9	60.8	647.3	2.2	40.7	64.0	61.5	69.9	60.6	64.7	42.1	466.6

Appendix C **Plasma Methionine (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

Initial	NAC1									NAC2								
	Time mins								AUC	*Time mins*								AUC
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	5.71	4.11	5.68	4.04	2.91	4.20	4.85	4.44	38.6									
PFN	6.42	7.43	6.19	5.82	5.62	5.57	6.34	6.91	55.7									
EGrY	6.39	6.04	6.00	5.96	3.34	4.57	4.16	4.22	41.1									
MHN	5.93	4.97	5.12	6.55	4.45	6.23	5.11	8.81	53.8									
AJN	5.28	5.25	3.29	3.13	3.80		3.98	7.43	40.4									
GKY	4.29	6.35	3.92	3.27	4.10	4.22	3.50	4.42	36.5									
NST	13.44	12.46	13.86	13.30	12.71	10.83	9.55	13.10	106.3									
OSN	5.32	5.76	8.42	8.53	7.14	7.72	6.49	4.95	61.3									
mean	6.6	6.5	6.6	6.3	5.5	6.2	5.5	6.8	54.6									
sd	2.8	2.6	3.3	3.4	3.2	2.4	2.0	3.1	22.9									
RMcE	4.97	5.16	4.05	4.98	4.29	4.35	4.18	3.89	39.0	6.93	5.43	3.95	3.75	2.77	3.10	5.04	4.87	37.6
EGY	3.66		6.33		3.23	4.23	4.58	3.95	39.6	3.39	3.37	3.26	4.24	3.60	3.67	3.49	3.00	31.7
DHD		2.36	4.60		1.85		2.25	2.76		4.41	8.58	4.77	6.16	2.96	3.22	3.38	4.41	37.4
PHS		no	results		avail able						no	results		avail able				
CKN	5.21	5.50	3.74		2.76	3.69	5.15	3.75	36.3	2.83	4.77	3.57	3.00	2.25		3.15	6.15	31.6
EMcE	5.10	6.44	4.48	3.09	3.97	5.09	5.80	4.59	43.4	5.53	5.47	4.15	3.67	5.19	4.07	2.79	3.46	35.2
JMK	5.37	6.57	3.62	5.11	3.77	2.21	2.02	2.13	28.3	4.97	8.06	4.79	5.30		3.39	2.14	2.82	33.6
SWS		5.37	6.71		3.88	5.13	3.98	4.05			no	results		avail able				
PFH	4.19	5.98	5.38	3.76	5.14	6.77	2.80			not	rechall enged			with drew				
mean	4.8	5.3	4.9	4.2	3.6	4.5	3.8	3.6	36.7	4.7	5.9	4.1	4.4	3.4	3.5	3.3	4.1	34.5
sd	0.7	1.4	1.2	1.0	1.0	1.4	1.4	0.8	5.6	1.5	2.0	0.6	1.2	1.1	0.4	1.0	1.3	2.7
JCN	3.87	2.68	2.12	3.63	1.51	2.21	2.06	2.39	20.8		no	results		avail able				
JGH	6.90	8.42	7.94	6.66	4.98	3.93	5.15	4.60	48.4		no	results		avail able				
NHN		no	results		avail able					not	rechall enged			with drew				
LJS	4.67	3.36	3.78	4.16	5.80	3.75	5.38	2.78	39.5		no	results		avail able				
GMN	2.88	2.65	3.88	2.63	2.72	2.23	1.82	1.60	20.5	1.71	2.63	2.77	1.91	1.58		1.92	2.19	17.6
POE	1.85	2.11	1.94	1.86	1.53	1.90	1.68	2.01	16.3	1.21	1.50	0.80	1.70	0.67	0.75	1.05	0.73	8.7
EPY	3.76	4.93	3.78	2.91	3.32	3.61	3.19	5.57	34.1		no	results		avail able				
mean	4.0	4.0	3.9	3.6	3.3	2.9	3.2	3.2	29.9	1.5	2.1	1.8	1.8	1.1	0.8	1.5	1.5	12.4
sd	1.7	2.4	2.2	1.7	1.8	0.9	1.7	1.6	12.7	0.4	0.8	1.4	0.1	0.6		0.6	1.0	6.3

Appendix C **Plasma GSH (μmol/l) and AUC (μmol/l/hr) after oral NAC loading in challenge subjects**

Initial	METH1								AUC	METH2								AUC
	Time mins									*Time mins*								
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	3.85	4.36	4.36	3.84	4.79	5.20	5.70	5.62	45.4									
PFN	5.83	5.66	5.85	8.48	6.18	5.08	5.81	5.80	54.4									
EGrY	6.45	7.51	6.81	8.56	4.96	3.45	3.97	6.59	48.8									
MHN	3.15	3.42	3.59		3.14	3.39	2.39	2.39	26.5									
AJN	4.95	4.89	6.24	6.49	7.63	8.76	8.44	6.73	67.0									
GKY	5.07	5.46	6.93	6.85	7.18	7.16	6.14	8.04	61.7									
NST	10.45	13.42	13.68	16.78	12.04	12.58	11.50	14.02	116.8									
OSN	4.41	4.24	5.04	4.75	8.86	4.77	7.22	4.67	54.2									
mean	5.5	6.1	6.6	8.0	6.8	6.3	6.4	6.7	59.9									
sd	2.2	3.2	3.1	4.3	2.8	3.1	2.8	3.4	26.2									
RMcE	7.31	5.99	5.35		6.27		6.63			4.20	4.41	3.30	6.90		2.94	5.27	4.13	41.6
EGY	3.58	7.27	4.96	5.05	3.83	3.42	3.37	3.08	35.1	3.35		4.61	4.67	4.87	3.11	2.95	4.01	34.4
DHD	3.34	3.74			4.53	2.90		6.56	39.0	3.26	4.39	2.62	5.24			3.61	3.07	35.6
PHS		no	results		avail able						no	results		avail able				
CKN	4.63		5.18	3.06	2.97	3.54	3.64	3.83	33.0	3.87	2.85	3.17	3.32	3.41	2.57	3.74	4.68	31.7
EMcE	8.34	6.14	6.31	7.21	8.10	4.07	5.23	5.02	53.1	3.58	4.31	4.98	4.20		4.21	4.24	3.79	37.6
JMK	5.14	4.03	3.02	4.15	6.60	3.23	3.05	2.29	34.0	2.49	3.88	4.15	4.93	2.53		1.71	3.28	25.3
SWS	4.22				4.36	5.34	4.45	2.99	38.6		no	results		avail able				
PFH	3.80	4.40	4.06	4.70	4.20	3.20	3.16	3.44	33.3	not	rechall enged			with drew				
mean	5.0	5.3	4.8	4.8	5.1	3.7	4.2	3.9	39.6	3.5	4.0	3.8	4.9	3.6	3.2	3.6	3.8	33.7
sd	1.8	1.4	1.1	1.5	1.7	0.8	1.3	1.5	7.1	0.6	0.7	0.9	1.2	1.2	0.7	1.2	0.6	5.5
JCN	2.49	3.39	2.27	1.69	1.90	2.07	2.16	1.81	18.7		no	results		avail able				
JGH	3.43	5.72	3.45	3.95		4.16	4.40	4.39	38.2		no	results		avail able				
NHN		no	results		avail able					not	rechall enged			with drew				
LJS	6.10	8.64	5.89	5.79	6.24	6.29	5.78	5.86	55.2		no	results		avail able				
GMN	4.35	4.90	6.30	4.26	3.83	2.25	1.85	1.49	26.5	2.25	2.31	2.61			2.63	1.41	0.74	17.8
POE	1.33	0.96	1.01	1.02	1.16	1.51	1.40	1.30	11.4	1.73	1.64	1.84	1.63	1.62	2.30	1.63	2.06	16.4
EPY	3.42	5.96	5.10	5.79	5.68	6.36	5.40	6.46	52.1		no	results		avail able				
mean	3.5	4.9	4.0	3.8	3.8	3.8	3.5	3.6	33.6	2.0	2.0	2.2	1.6	1.6	2.5	1.5	1.4	15.9
sd	1.6	2.6	2.1	2.0	2.2	2.2	1.9	2.3	17.8	0.4	0.5	0.5			0.2	0.2	0.9	1.0

Appendix C **Plasma GSH (μmol/l) and AUC (μmol/l/hr) after oral Methionine loading in challenge subjects**

Initial	NAC1								AUC	NAC2								AUC
	Time mins									*Time mins*								
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	10.21	9.96	9.54	9.52	9.25	9.78	9.72	9.64	86.7									
PFN	12.12	11.37	11.92	11.10	11.29	11.38	11.09	10.92	101.1									
EGrY	9.43	8.52	9.65	9.43	9.82	9.63		9.27	85.4									
MHN	8.45	8.03	8.32	7.93	8.42	7.84	8.00	8.72	73.7									
AJN	6.04	6.15	6.28	6.23	6.24	6.62	5.97	5.88	55.5									
GKY	8.37	8.12	8.15	8.06	8.73	8.70	8.98	8.87	77.9									
NST	8.07	7.81	7.83	7.64	8.48	7.76	8.86	8.09	73.9									
OSN	9.64	9.62	9.42	9.70	9.41	9.83	9.62	9.44	86.3									
mean	9.0	8.7	8.9	8.7	9.0	8.9	8.9	8.9	80.1									
sd	1.8	1.6	1.7	1.5	1.4	1.5	1.6	1.5	13.3									
RMcE	7.81	7.90	8.17	7.71	7.57	7.82	7.56	7.68	69.3	4.72	5.05	5.29	4.98	4.82	5.27	5.61	5.00	46.6
EGY	10.05	9.82	10.27	10.07	9.17	9.10	9.66	8.23	84.1	4.99	4.34	5.06	4.58	4.50	6.10	5.01	4.88	45.0
DHD	4.08	4.22	3.86	4.00	4.58	3.90	3.89	4.47	37.2	8.30	8.44	8.63	7.81	7.62	7.79	8.05	8.75	72.8
PHS	9.54	9.89	9.44	9.69	9.80	11.31	8.49	9.86	87.3	10.37	10.64	10.80	10.64	9.97	11.09	10.32	9.90	93.6
CKN	4.66	6.02	4.57	4.84	4.64	4.69	5.21	4.44	43.7	8.03	8.04	11.56	8.76	8.17	8.33	8.03	8.02	75.4
EMcE	4.34	3.63	3.71	3.44	3.05	3.68	3.37	3.77	31.5	9.08	9.16	9.28	8.89	8.63	9.34	9.99	9.04	83.4
JMK	3.48	3.60	3.60	3.40	3.35	3.22	3.09	3.15	29.4	7.38	7.28	7.24	7.79	7.41	7.31	7.12	7.21	65.8
SWS	8.44	8.26	8.36	8.67	9.05	8.07	8.43	8.52	76.5	8.41	8.28	8.45	8.37	8.26	8.25	8.19	7.83	73.8
PFH	8.94	9.27	9.34	9.68	10.29	9.51	9.64	8.95	86.0	not	rechall	enged	with	drew				
mean	6.8	7.0	6.8	6.8	6.8	6.8	6.6	6.6	60.6	7.7	7.7	8.3	7.7	7.4	7.9	7.8	7.6	69.5
sd	2.6	2.6	2.8	2.9	2.9	3.0	2.7	2.6	24.7	1.9	2.1	2.3	2.0	1.9	1.8	1.9	1.8	16.8
JCN	10.24	9.99	9.86	10.60	9.78	9.79	9.19	9.47	87.3	8.97	9.05	8.57	8.26	8.46	8.7	8.88	8.34	77.5
JGH	8.24	9.30	9.10	8.81	9.20	9.12	9.86	7.99	81.7	9.22	9.81	9.4	8.69	8.36	9.02	9.64	9.66	82.9
NHN	5.71	6.96	6.64	6.78	6.31	7.53	7.09	7.38	62.8	not	rechall	enged	with	drew				
LJS	10.83	11.57		11.26	10.52	12.10	10.98	10.93	100.5	9.01	8.91	8.17	9.25	8.82	9.46	9.46	9.88	83.6
GMN	6.70	7.39	6.84	7.34	7.09	7.21	8.09	7.94	67.7	9.22	8.69	8.63	8.67	8.39	8.50	9.12	9.18	79.3
POE	4.42	4.04	3.92	4.28	3.80	4.29	4.49	3.90	37.5	6.91	7.74	9.44	7.56	7.22	7.62	7.38	6.98	67.2
EPY	8.06	7.86	6.87	6.26	7.08	5.58	6.38	9.03	62.5	9.57	8.99	8.8	9.39	9.52	9.17	9.9	9.65	85.5
mean	7.7	8.2	7.2	7.9	7.7	7.9	8.0	8.1	71.4	8.8	8.9	8.8	8.6	8.5	8.7	9.1	8.9	79.3
sd	2.3	2.4	2.1	2.5	2.3	2.6	2.2	2.2	20.5	1.0	0.7	0.5	0.7	0.7	0.6	0.9	1.1	6.6

Appendix C **Whole blood GSH (μmol/gHb) and AUC (μmol/gHb/hr) after oral NAC loading in challenge subjects**

Initial	METH1								AUC	METH2								AUC
	Time mins									*Time mins*								
	0	30	60	90	180	270	360	540		0	30	60	90	180	270	360	540	
HBS	9.72	9.10	9.22	9.24	9.03	9.03	8.86	8.91	81.2									
PFN	12.26	11.78	10.25	10.37	10.82	10.07	10.50	10.59	95.3									
EGrY	9.19	9.65	8.91	9.04	8.93	9.62	9.45	9.59	84.1									
MHN	8.95	8.36	8.59		8.16	7.81	9.94	8.11	77.7									
AJN	5.98	5.63	6.44	6.08	6.41	5.86	6.10	6.30	55.2									
GKY	7.75	7.72	7.95	7.73	7.78	7.81	7.73	7.32	69.3									
NST	8.39	8.58	8.56	8.46	8.52	8.10	8.85	8.18	76.2									
OSN	10.96	8.75	10.05	10.71	11.34	12.65	10.34	10.72	98.2									
mean	9.2	8.7	8.7	8.8	8.9	8.9	9.0	8.7	79.6									
sd	1.9	1.7	1.2	1.6	1.6	2.0	1.5	1.5	13.8									
RMcE	7.82	7.63	7.83	7.40	8.07	8.47	7.92	7.90	71.6	8.34	8.41	7.91	8.64	8.60	8.64	8.88	7.97	76.7
EGY	7.28	9.07	8.25	8.51	8.05	7.31	8.59	7.77	73.0	8.52	8.38	9.09	9.65	8.24	8.41	9.16	8.28	78.5
DHD	8.12	7.49	7.92	8.00	8.14	7.03	8.03	7.74	70.2	8.95	8.69	9.27	9.34	9.73	8.66	9.54	8.56	82.4
PHS	8.27	8.25	8.35	7.17	7.86	7.36	8.32	9.79	73.8	9.88	8.71	9.14	9.66	9.05	8.70	8.73	8.77	80.5
CKN	6.66	5.50	9.22	6.61	6.63	7.25	6.57	6.81	61.5	8.82	8.53	8.79	8.60	9.00	8.69	8.82	8.93	79.2
EMcE	8.95	8.70	8.29	8.04	7.99	8.31	8.08	9.28	75.3	9.05	8.47	9.33	8.09	7.98	8.89	8.26	8.38	75.7
JMK	6.85	7.18	6.62	7.04	7.54	7.56	6.50	7.17	63.7	5.69	5.39	4.85	4.57	5.52	4.99	4.61	5.84	46.0
SWS	9.79	10.02	10.04	9.50	9.69	9.79	9.58	9.65	87.2	6.47	5.81	6.92	6.64	6.14	5.79	6.62	6.75	57.5
PFH	8.76	8.63	9.53	8.96	9.53	9.40	9.18	8.66	82.3	not	rechall enged		with drew					
mean	8.1	8.1	8.5	7.9	8.2	8.1	8.1	8.3	73.2	8.2	7.8	8.2	8.1	8.0	7.8	8.1	7.9	72.1
sd	1.0	1.3	1.0	1.0	0.9	1.0	1.0	1.1	8.1	1.4	1.4	1.6	1.8	1.5	1.5	1.7	1.1	13.1
JCN	9.34	9.50	9.12	9.22	8.89	8.84	8.83	7.27	78.2	9.35	8.64	9.11	8.91	9.32	10.76	9.13	9.09	84.4
JGH	9.08	8.97	8.90	9.68	9.73	8.08	8.94	9.33	81.7	10.09	9.88	10.31	9.63		10.62	10.64	10.47	93.0
NHN	6.75	7.16	7.21	6.94	6.55	7.28	7.37	7.95	65.1	not	rechall enged		with drew					
LJS	10.94	11.26	12.08	10.68	11.14	11.32	11.31	10.95	100.6	10.64	10.81		10.31	10.28	10.94	12.08	10.64	98.6
GMN	7.81	8.32	8.87	8.74	10.67	8.42	9.20	9.75	83.2	9.79	8.48	9.24		10.54	8.32	8.39	8.06	80.1
POE	7.11	6.95	7.41	6.70	6.82	6.86	7.24	6.55	62.3	6.93	8.29	6.64	6.77	6.85	7.09	6.92	7.60	63.8
EPY	8.35	7.44	7.32	7.57	8.31	7.85	8.49	7.96	72.3	8.17	8.46	8.31	7.83	8.53	10.58	10.21	11.77	87.6
mean	8.5	8.5	8.7	8.5	8.9	8.4	8.8	8.5	77.6	9.2	9.1	8.7	8.7	9.1	9.7	9.6	9.6	84.6
sd	1.4	1.5	1.7	1.5	1.8	1.5	1.4	1.5	12.9	1.4	1.0	1.4	1.4	1.5	1.6	1.8	1.6	12.1

Appendix C **Whole blood GSH (μmol/gHb) and AUC (μmol/gHb/hr) after oral Methionine loading in challenge subjects**

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