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STUDIES ON INORGANIC PYROPHOSPHATE IN PYROPHOSPHATE ARTHROPATHY

BY

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ABSTRACT

Age, metabolic disease and familial tendency all predispose to CPPD crystal deposition. However, the effects of these predisposing factors on articular PPi metabolism have not been investigated.

The activities of the synovial fluid enzymes, NTPP, 5NT and ALP have previously been implicated in the pathogenesis of pyrophosphate arthropathy and CPPD crystal deposition. Normal controls, however, are lacking in previous studies. Whether a true relationship exists between these synovial fluid enzymes and crystal deposition, therefore, is unclear.

Reduced synovial fluid PPi concentrations have been reported in acute pseudogout compared to chronic pyrophosphate arthropathy, suggesting an association between inflammation and PPi concentrations. Few studies, however, have characterised joints according to inflammatory state. The effects of the inflammatory state of the joint on PPi metabolism, including effects on the activities of the enzymes NTPP, ALP and 5NT, are therefore, of interest in their putative association with CPPD crystal deposition.

In familial pyrophosphate arthropathy a systemic disorder of PPi metabolism has been proposed. In the sporadic form of pyrophosphate arthropathy, however, whether altered PPi metabolism reflects a generalised abnormality of cartilage in predisposed individuals, or a localised response to joint damage has not been investigated.

In this study articular PPi metabolism was studied in conditions that predispose to CPPD deposition, by the measurement of synovial fluid PPi and the activities of the enzymes NTPP, ALP and 5NT, which could be responsible for an aberration in articular PPi metabolism leading to CPPD crystal deposition. In addition knees were classified according to their clinical inflammatory state preceding aspiration to assess the effects of disease activity on articular PPi metabolism.

Knee synovial fluid PPi levels and NTPP activity were elevated in pyrophosphate arthropathy compared to osteoarthritis, rheumatoid arthritis and normal, implicating an error in PPi metabolism promoting CPPD crystal deposition. Knee synovial fluid PPi levels showed a positive correlation with NTPP activity in all disease groups, implicating NTPP as a major source of synovial fluid PPi. No correlation was apparent between age and synovial fluid PPi levels or NTPP activity in normals, despite the strong association between ageing and CPPD crystal deposition.

The effects of clinical inflammation varied between the disease groups studied, affecting PPi metabolism in pyrophosphate arthropathy and rheumatoid arthritis but not in osteoarthritis, suggesting the existence of different inflammatory mechanisms between diseases.

The enzymes ALP and 5NT, which have been implicated in the promotion of CPPD crystal deposition, via effects on PPi metabolism, showed no such association in this study, but did relate to the inflammatory state of the joint.

A defect in articular PPi metabolism was evident in several of the metabolic diseases studied which are known to predispose to CPPD crystal deposition. In haemochromatosis, hypomagnesaemia and hyperparathyroidism, increased synovial fluid PPi was evident. Synovial fluid NTPP activity was similarly increased in haemochromatosis and hypomagnesaemia.

Studies on articular PPi metabolism in familial pyrophosphate arthropathy did not reveal any abberation in PPi metabolism compared to the sporadic form of the disease. Factors promoting CPPD crystal deposition, in addition to those affecting PPi metabolism may be under genetic influence.

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ABBREVIATIONS.

ADA	adenosine deaminase
ALP	alkaline phosphatase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ca	calcium
cAMP	cyclic adenosine monophosphate
CaCl	calcium chloride
CPPD	calcium pyrophosphate dihydrate
Cu	copper
DABA	3,5-diaminobenzoic acid
DNA	deoxyribonucleic acid
FCS	fetal calf serum
Fe	iron
GLDH	glutamate dehydrogenase
HCl	hydrogen chloride
KCl	potassium chloride
MEM	minimum essential medium
Mg	magnesium
MgCl	magnesium chloride
$MgSO_4$	magnesium sulphate
NAD	nicotinamide adenine dinucleotide (oxidised)

NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide dinucleotide phosphate
NaH_2 PO_4	sodium hydrogen phosphate
NMP	nucleoside monophosphate
NTP	Nucleoside triphosphate
NTPP	nucleoside triphosphate pyrophosphatase
5NT	5'nucleotidase
PBS	phosphate buffered saline
PPi	inorganic pyrophosphate
PTH	parathyroid hormone
TCA	trichloroacetic acid
TSH	thyroid stimulating hormone
UDPG	uridine diphosphoglucose
UTP	uridine triphosphate

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CHAPTER 1: Introduction.

1.1 Pyrophosphate arthropathy.

1.1.1 Historical background.

Crystals were first implicated as causative agents in joint disease in 1859, when Sir Alfred Baring Garrod suggested that the crystalline deposits in gout were the cause and not the effect of gouty inflammation (Garrod 1859). Subsequently, with the development of sophisticated analytical techniques, a variety of intra-articular deposited crystalline salts have been identified. Calcium phosphate and calcium pyrophosphate dihydrate (CPPD) are the two main non-urate crystals found to deposit in joints. Like urate crystals, their presence is implicated in the pathogenesis of specific clinical syndromes, allowing the distinction between these crystal arthropathies.

McCarty and Hollander (1961) identified CPPD crystals for the first time in 1961, when examining the synovial fluid from patients thought to be suffering from acute gout. With the aid of polarised light microscopy, and later x-ray diffraction, They were able to distinguish CPPD crystals from monosodium urate monohydrate found in gout (Kohn *et al* 1962). This new crystal-induced disease was named calcium pyrophosphate dihydrate crystal deposition disease, also termed pyrophosphate arthropathy.

Pyrophosphate arthropathy predominantly occurs in the elderly as either: a chronic symptomatic arthritis with structural change, or an acute self-limiting synovitis (Doherty and Dieppe 1988). The latter is referred to as pseudogout due to the similarity with the clinical features of gout (McCarty *et al* 1962).

A common radiological finding in pyrophosphate arthropathy is chondrocalcinosis, exhibited in figure 1, which refers to the detection of calcium salts deposited in particularly the menisci and articular cartilage of the knee. Such salts have most commonly been identified as CPPD, although other calcium salts have been detected (McCarty *et al* 1966(a), Mitrovic *et al* 1982). Although chondrocalcinosis is a prominent feature of pyrophosphate arthropathy, it exists more commonly as an isolated feature in the absence of any co-existing joint disease. The prevalence of chondrocalcinosis increases dramatically with age, occurring in 30-60% of people aged over 85 years (Ellman *et al* 1981). In addition to chondrocalcinosis, another radiographic feature prominent in pyrophosphate arthropathy, is a unique distribution of affected joints, involving knees, ankles, shoulders, wrists, and metacarpophalangeal joints, involvement of the spine, hip and elbow also occurs, although less frequent. This feature distinguishes this crystal subset from uncomplicated osteoarthritis (Doherty 1983). The diversity of clinical expressions apparent in pyrophosphate arthropathy implies that several factors are involved in the pathogenesis of the disease, and in respect of asymptomatic chondrocalcinosis, it would appear that initiating factors are involved in disease onset

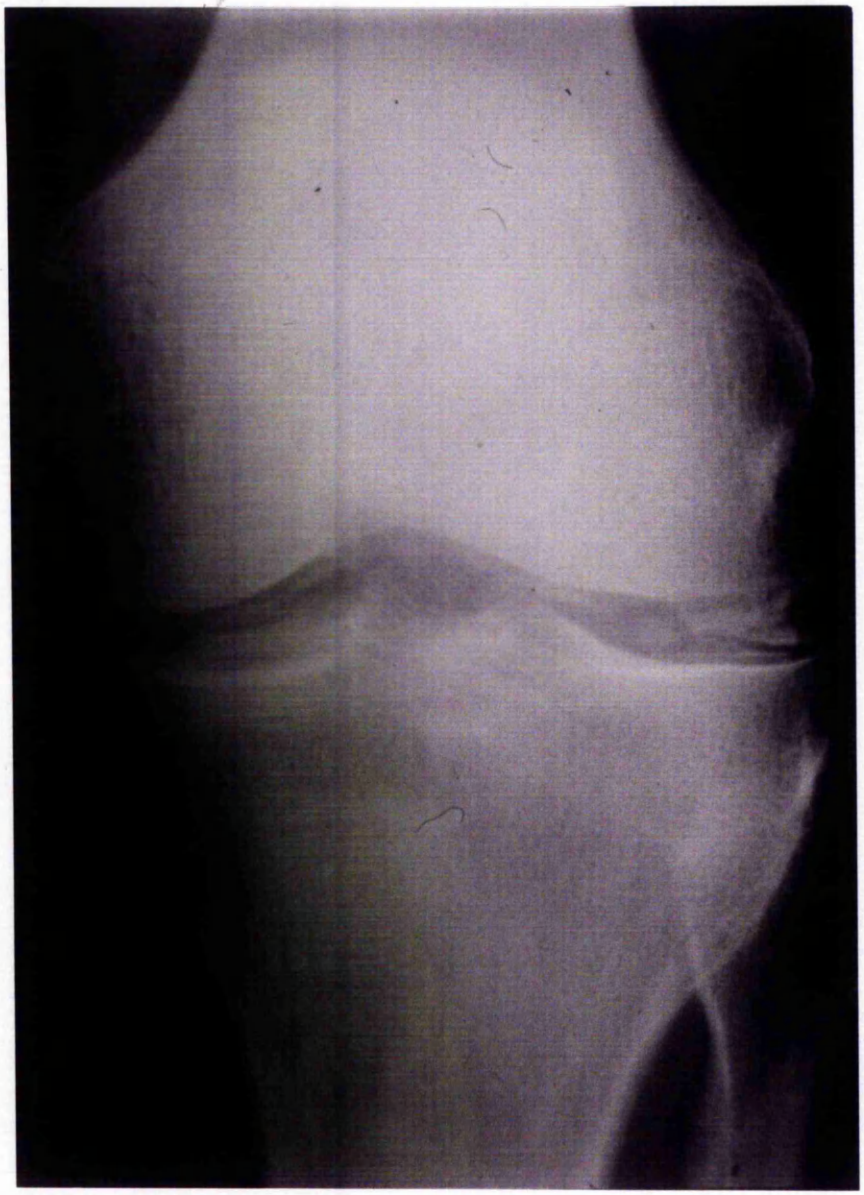


Figure 1: Radiographic demonstration of chondrocalcinosis in the knee joint.

1.1.2 Predisposing factors to calcium pyrophosphate dihydrate crystal deposition.

Pyrophosphate arthropathy commonly exists as a sporadic finding, increasing with age (Ellman and Levin 1975, Ellman *et al* 1981, Wilkins *et al* 1982) and possible previous joint injury or trauma (Doherty *et al* 1982, De Lange and Keats 1985, Linden and Nilssen 1978). A more unusual finding is the familial form of the disease, which differs from sporadic pyrophosphate arthropathy in commonly presenting at an early age as a severe polyarticular disease (Zitnan and Sitaj 1976).

Several metabolic disease associations have also been postulated, as described in table 1. However, it would seem that some are no more than coexisting age relationships. Despite this, some metabolic disorders do seem to exhibit a true association, where the deposition of CPPD crystals may be an expression of the metabolic disturbance involved. Mechanisms involved in such associations are unclear, however, a disturbance in inorganic pyrophosphate (PPi) metabolism has been suggested in several instances, for example in hypophosphatasia, hyperparathyroidism, hypomagnesaemia and haemochromatosis (McGuire *et al* 1980(a), Doherty *et al* 1991(a)).

Postulated mechanisms responsible for the deposition of CPPD crystals are varied. As histological evidence favours hyaline and fibrocartilage as the sites of deposition (Schumacher 1976) a disturbance within the cartilage is implicated.

Predisposing factors:		Possible mechanisms involved:
Genetic predisposition	-	Error in PPi metabolism Alteration of cartilage matrix
Ageing	-	Altered PPi metabolism Altered cartilage matrix; increase in nucleating factors or decreased inhibitors of nucleation
Metabolic disease: Hypophosphatasia Haemochromatosis Hypomagnesemia Hyperparathyroidism Hypothyroidism Wilson's disease Ochronosis	-	Altered PPi metabolism Metabolic changes in cartilage Increased extracellular calcium Increased crystal nucleators
Other joint diseases: Osteoarthritis Gout Amyloid arthropathy Neuropathic joints	-	Epitaxy on apatite or urate crystals Cartilage damage Alteration of cartilage matrix

Table 1: Possible predisposing factors to CPPD crystal deposition

Increased local concentrations of either calcium or PPi could lead to CPPD crystal deposition. Although no increases in local calcium have been found, except in hyperparathyroidism, much evidence supports a local disturbance of PPi metabolism in pyrophosphate arthropathy (Rachow and Ryan 1988). Increased concentrations of PPi have been reported in pyrophosphate arthropathy synovial fluid (Russell *et al* 1970, McCarty *et al* 1971, Altman *et al* 1973(a), Silcox *et al* 1973) and PPi is generated by osteoarthritic and chondrocalcinotic cartilage (Howell *et al* 1975). A systemic disturbance of PPi metabolism has been reported in hereditary pyrophosphate arthropathy (Lust *et al* 1981, Ryan *et al* 1986). The cause of such a disturbance of PPi metabolism may be influenced by joint damage, metabolic disorders, or ageing alone.

Changes occurring in the cartilage matrix with age or with the onset of disease could also promote crystallisation. Matrix changes could act by decreasing inhibitors of crystal nucleation and growth, or by increasing promoters. Nucleating factors may be the presence of other crystal species, for example apatite or urate crystals, or ions which may exist in excess such as, iron salts in haemochromatosis (Hearn *et al* 1978).

CPPD crystals, by shedding from the articular or fibrocartilage into the synovial space, produce the acute attacks associated with pseudogout (McCarty *et al* 1966(a)). The mechanisms behind such shedding are varied and include: damage to the joint in the form of injury or existing disease (Bennett *et al* 1974),

enzymatic damage to the cartilage (Smith and Phelps 1972), metabolic change in cartilage (Dorwart and Schumacher 1975) and major surgery (O'Duffy 1976). The self-limiting nature of acute attacks is ill-defined, although several mechanisms have been suggested: removal of crystals by phagocytosis, dissolution of crystals, anti-inflammatory activity and alteration of the crystal surface (Dieppe and Doherty 1982). Ironically, despite the phlogistic nature of CPPD crystals in acute pseudogout and *in vivo* experiments (McCarty *et al* 1962, McCarty *et al* 1966(b)) CPPD crystal deposition commonly exists in some individuals in the absence of joint disease (Ellman and Levin 1975). The role of these crystals in chronic pyrophosphate arthropathy, therefore, is uncertain.

It is unclear whether CPPD crystals are causative agents contributing to the degeneration of cartilage and subsequent arthritis, a product of pre-existing joint disease, or "innocent bystanders". It is clear, however, that factors in addition to CPPD crystals within the cartilage or synovial fluid are important in the pathogenesis of pyrophosphate arthropathy.

1.2 Inorganic pyrophosphate metabolism.

Inorganic pyrophosphate (PPi) is known to be produced during many biosynthetic reactions. The major source is thought to be from pyrophosphorylysis of nucleoside triphosphates, during the biosynthesis of most major cell macromolecules,

including: proteins, lipids, phospholipids, nucleotides, nucleic acids, urea, steroids, structural polysaccharides, and glycogen (Russell 1976). It is thought that the quantity of PPi produced as a result of such reactions is in the order of kilograms daily. Removal of PPi is therefore, of major physiological importance. Extracellular PPi, found in plasma, saliva, synovial fluid and other body fluids, is thought to be entirely endogenous in origin as dietary PPi is completely hydrolysed to Pi within the intestine by alkaline phosphatase (ALP) (Russell 1976). In view of the inability of PPi to cross the cell membrane (Felix and Fleish 1977), the source of this extracellular PPi is uncertain.

The hydrolysis of PPi to Pi appears to be the most common mechanism of removal of intracellular PPi. This is catalysed by pyrophosphatases including glucose-6-phosphatase, ALP and non-specific pyrophosphatases. ALP is thought to account for the hydrolysis of up to 80% of extracellular PPi. Hydrolysis of PPi was shown to account for at least 25% of removal in dogs, whilst urinary excretion accounted for only 10% (Jung *et al* 1970). Initially, due to the finding of very low intracellular concentrations of PPi, the hydrolysis of PPi by pyrophosphatases was thought to be in equilibrium, pulling reactions in the direction of biosynthesis (Kornberg 1962). Subsequent findings of measurable quantities of intracellular PPi in rat liver (Flodgaard and Feron 1974) and biological fluids, however, have questioned this initial hypothesis and suggested that PPi hydrolysis is controlled by effects on pyrophosphatase activity.

Intracellular PPi content has also been found to vary with cell type. Chondrocytes, for example, contain higher PPi concentrations than synovial cells or fibroblasts (Caswell *et al* 1983). In addition the PPi content and output of some cells is under the influence of external stimuli; for example intracellular PPi in liver varies with different nutritional states (Lawson *et al* 1976), suggesting a controlling effect of PPi on metabolic pathways. PPi may also exert phenotypic regulation over some cell types; for example, addition of PPi to chondrocyte cultures caused the switching of collagen synthesis from type II to type I (Deshmukh and Sawyer 1978), again demonstrating a regulatory role for this ion. Other putative roles for intracellular PPi have been postulated including effects on intracellular Ca^{++} (Verces and Lehniger 1984), enzyme activities (Dancker 1983) and transport of nucleotides (D'Souza and Wilson 1982, Kramer 1985) and iron (Nilsen and Romslo 1984, Cheuk *et al* 1987.

Although it is not known what controls intracellular or extracellular PPi levels, several disease states exist where a disorder of PPi metabolism is apparent. In familial pyrophosphate arthropathy, intracellular PPi was found to be elevated in transformed lymphoblasts and fibroblasts (Lust *et al* 1981, Ryan *et al* 1986), suggesting a defect in one or more metabolic pathways. Extracellular PPi metabolism is disturbed in hypophosphatasia, where a deficiency of ALP leads to a four-fold increase in plasma and serum PPi levels. Plasma PPi is also elevated in renal failure and in some cases of acromegaly and osteomalacia (Russell *et al*

1971, Silcox and McCarty 1973, Camerlain *et al* 1980). However, in sporadic pyrophosphate arthropathy and osteoarthritis, a systemic increase in PPi is not evident, plasma and urinary levels being normal (Pflug *et al* 1969, Russell *et al* 1970, Ryan *et al* 1979(a), Camerlain *et al* 1980).

1.3 Synovial fluid inorganic pyrophosphate.

A disturbance in PPi metabolism in pyrophosphate arthropathy was initially implicated when Russell (1970) discovered that synovial fluid PPi concentrations in patients with pyrophosphate arthropathy were elevated in comparison to other arthritic diseases. In respect of previous normal PPi concentrations found in the urine (Pflug *et al* 1969) and plasma (Russell *et al* 1970), a local rather than systemic disturbance of PPi was proposed. Calcium, in contrast was found to be low in pyrophosphate arthropathy synovial fluid (Russell *et al* 1970) or similar to control synovial fluid (McCarty *et al* 1971, Altman *et al* 1973). Increased synovial fluid PPi in pyrophosphate arthropathy was later confirmed by several groups (McCarty *et al* 1971, Altman *et al* 1973(a), Silcox *et al* 1974) with the additional finding that synovial fluid PPi is also elevated in osteoarthritis compared to rheumatoid arthritis and normals.

Low synovial fluid PPi concentrations in rheumatoid arthritis and acutely inflamed joints support increased PPi clearance from the joint with increased inflammation

(Camerlain *et al* 1975), probably due to increased synovial blood flow and synovial fluid pyrophosphatases. Synovial fluid PPi has been shown to correlate with the degree of radiographic degeneration of the joint (Silcox and McCarty 1974) supporting the association between joint damage and crystal deposition.

Postulated mechanisms leading to a localised increase in synovial fluid PPi include: reduced removal of PPi via venous blood and lymphatics; impaired hydrolysis of PPi, due to reduced activity of pyrophosphatases; leakage from cartilage or synovium; or increased biosynthesis of PPi from local tissue (Howell 1985). The dissolution of crystals is thought to be an unlikely contributor to synovial fluid PPi (Camerlain *et al* 1975) due to the high turnover rate of PPi in the joint, and the very slow dissolution rate of crystals in synovial fluid. Solubility conditions *in vivo*, however, may differ.

1.4 Articular cartilage inorganic pyrophosphate production.

The localised increase of PPi in the synovial fluid found in pyrophosphate arthropathy, suggests increased output of PPi by one or more intra-articular tissues. As histological evidence favours hyaline and fibrocartilage as the initial sites of CPPD crystal deposition (McCarty *et al* 1966(a), Boivin and Lagier 1983), articular cartilage would seem the most likely source of synovial fluid PPi.

Howell and colleagues (1975) demonstrated that osteoarthritic and chondrocalcinotic cartilage in organ culture generate PPi, in comparison to normal cartilage and cartilage from hip fractures, avascular necrosis, rheumatoid arthritis, and non-articular elastic cartilage, all of which failed to generate measurable quantities of PPi. More recent studies utilising more sensitive methods of measuring PPi have demonstrated that normal cartilage in organ culture (Ryan *et al* 1981) and monolayer culture (Caswell and Russell 1985) elaborate PPi, suggesting that PPi production by articular cartilage is a normal phenomenon upregulated in joint disease. Other articular tissues including synovium (Howell *et al* 1975) and subchondral bone (Ryan *et al* 1981) have failed to elaborate any measurable PPi in culture. Subchondral bone was originally considered a potential PPi source, and could account for CPPD deposits in the mid-zone of cartilage where theoretically, the PPi from bone, and calcium from the joint fluid would meet. However, since bone adsorbs rather than releases PPi (Ryan *et al* 1981) this source is uncertain.

Postulated mechanisms by which PPi reaches the extracellular space are illustrated in figure 2. Increased extracellular PPi may originate from the intracellular space, either exported with matrix components synthesised by the chondrocytes or released after cell damage or death. Chondrocytes produce large quantities of proteoglycans which are exported to the cartilage matrix. Since PPi is produced as a by-product of proteoglycan synthesis (Seubert *et al* 1985) the hypothesis that PPi also accompanies the exportation of proteoglycans would seem a favourable

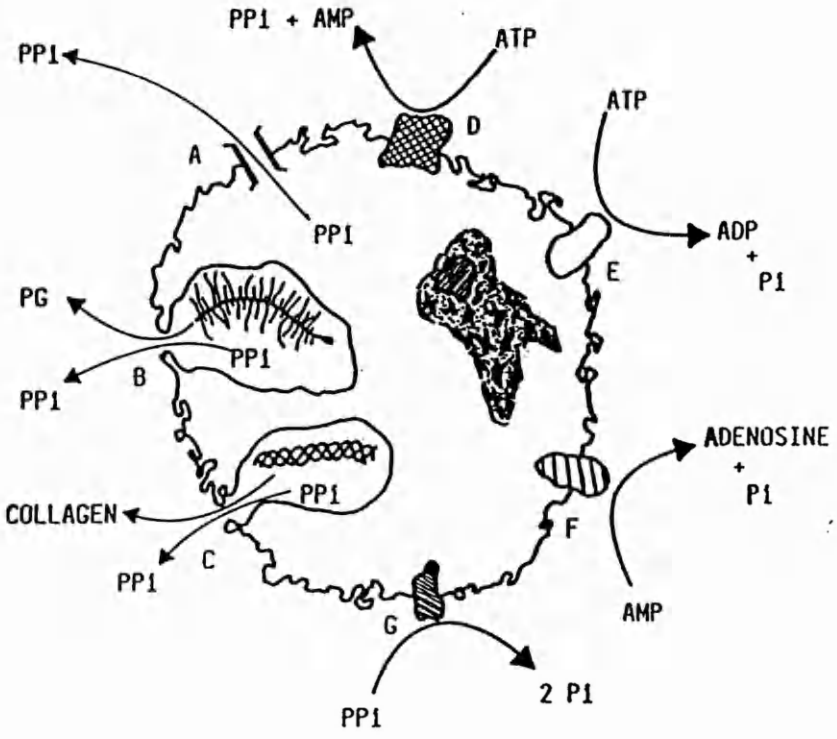


Figure 2: Postulated mechanisms of PPi elaboration by chondrocytes.

A. Unknown mechanisms may exist by which intracellular PPi may be transported across theplasma membrane.
B and **C.** PPi may be extruded from the chondrocyte as an "innocent bystander" along with PG or collagen.
D. Ecto-nucleoside triphosphate pyrophosphohydrolase could directly generate PPi from ATP.
E. Deficiency of ecto-ATPase would tend to shift ATP breakdown to pyrophosphohydrolysis (D).
F. Ecto 5'nucleotidase could promote PPi generation by removing the AMP formed by pyrophosphorylysis of ATP.
G. Deficiency of ecto-pyrophosphatase could contribute to extracellular accumulation of PPi.

PPi = inorganic pyrophosphate; Pi = inorganic orthophosphate; ATP, ADP, AMP, = adenosine tri, di, and monophosphate, respectively; PG = proteoglycan. (Rachow and Ryan 1988).

one. PPi release by lapine and canine but not human hyaline and fibro-cartilage in organ culture correlates with uronic acid production, indirectly suggesting PPi production accompanying increased production and exportation of proteoglycans (Ryan *et al* 1981). Ishikawa (1989) identified chondrocytes in the region of CPPD deposits unique in their increased content of proteoglycans. He suggested that such chondrocytes release these proteoglycans accompanied by PPi in the event of cell damage or death, leading to local CPPD crystal deposition.

More recent evidence, however, suggests that the connection between PPi and proteoglycan production is more tenuous than previously thought. The inhibition of proteoglycan synthesis and sulphation has been shown to have little or no effect on PPi production by chondrocytes in monolayer culture (Prins *et al* 1986) and cartilage in organ culture (Ryan *et al* 1990). As evidence is against PPi passively crossing the plasma membrane (Felix and Fleisch 1977), the co-secretion of PPi with macromolecules remains a favourable theory, if not with proteoglycans perhaps with some other macromolecule, for example collagen.

PPi could also reach the extracellular space by leakage from damaged or dying chondrocytes. Such leaky chondrocytes may exist in an osteoarthritic joint where cartilage damage is prevalent. Such an example associating previous joint damage with CPPD deposition was demonstrated by Doherty *et al* (1982) who found an increased incidence of crystal deposition in post-menisectomy knees.

Another important mechanism by which an excess of extracellular PPi may result is by the presence of ecto-enzymes on the chondrocyte membrane. Nucleoside triphosphate pyrophosphatase (NTPP) is an ecto-enzyme which catalyses the reaction:

$$NTP \rightarrow NMP + PPi$$

NTPP acts on extracellular nucleotides possibly leaked from damaged chondrocytes. NTPP activity is increased in cartilage and synovial fluid containing CPPD crystals compared to osteoarthritic and normal (Altman *et al* 1973(a), Tenenbaum *et al* 1981, Pattrick *et al* 1991).

With the additional increase in activity of another ecto-enzyme 5'nucleotidase (which catalyses the breakdown of AMP), in cartilage extracts, Tenenbaum and colleagues (1981) postulated that this increased removal of AMP would consequently increase PPi production. Adenylate cyclase has also been postulated as a possible source of PPi (Russell 1976). However, the active site of this enzyme faces intracellularly, and because of the inability of PPi to cross the plasma membrane unaided, this seems an unlikely source of extracellular PPi.

As well as increased production of PPi, decreased removal from the extracellular space is also pertinent. Alkaline phosphatase (ALP) and pyrophosphatase activities were found to be reduced in chondrocalcinotic cartilage extracts facilitating an excess of extracellular PPi (Howell *et al* 1976, Tenenbaum *et al* 1981).

1.5 Articular cartilage and synovial fluid nucleoside triphosphate pyrophosphatase activity.

Increased activity of the enzyme NTPP in chondrocalcinotic cartilage was initially demonstrated by Tenenbaum and colleagues (1981). The subsequent finding of increased NTPP activity in the synovial fluid from patients with pyrophosphate arthropathy (Rachow and Ryan 1985(a)) compared to osteoarthritis and rheumatoid arthritis has resulted in the implication of this enzyme in the pathogenesis of pyrophosphate arthropathy.

NTPP has been identified in several mammalian tissues including: rat (Decker and Bischoff 1972, Flodgaard and Torp-pedersen 1978), human (Lieberman *et al* 1967) and mouse (Evans 1973) liver plasma membrane; human plasma (Mills 1966); blood cells (Verhoef *et al* 1980); bovine epiphyseal cartilage (Hsu 1983); matrix vesicles (Siegel 1983. Caswell *et al* 1987); and human bone (Caswell and Russell 1988).

Various roles for NTPP have been postulated including calcium transport (Flodgaard and Torp-persen 1978); intracellular adhesion (Evans *et al* 1973); and recovery of nucleotides leaked into the extracellular space (Bischoff *et al* 1970, Decker 1972, Tran-Thi *et al* 1981). In the matrix vesicles of bovine and rabbit epiphyseal cartilage NTPP is thought to induce the initial stages of calcification by the production of PPi (Seigel *et al* 1983, Caswell *et al* 1987). In human,

porcine and canine articular cartilage NTPP has been identified as an ecto-enzyme (Ryan *et al* 1984, Howell *et al* 1984, Caswell and Russell 1985) with its active site facing outward on the chondrocyte. The ecto position of the enzyme on chondrocytes supports the putative role for NTPP together with 5'nucleotidase as a salvage pathway for nucleotides escaping from damaged or dying cells.

NTPP is present on normal human chondrocytes (Caswell and Russell 1985) and has the ability to hydrolyse large amounts of substrate. It has been postulated, therefore, that an excess of substrate may be important in the generation of PPi and subsequent CPPD crystal deposition (Ryan *et al* 1985). This theory is promoted by the finding of increased ATP concentrations in the synovial fluid of patients with pyrophosphate arthropathy compared to osteoarthritis and rheumatoid arthritis (Ryan *et al* 1987). Synovial fluid is thought to be in equilibrium with cartilage extracellular fluid, making this excess ATP a likely substrate for the chondrocyte ecto-enzyme. Such an increase in nucleoside triphosphates may result from cartilage damage with disruption of cell membranes and leakage of nucleoside triphosphates into the extracellular space. Adenine nucleotides are also known to be selectively released by cells in response to extracellular stimuli (Pearson and Gordon 1979), although whether this phenomena occurs in chondrocytes is unknown.

Synovial fluid NTPP activity, like cartilage NTPP, is increased in pyrophosphate arthropathy compared to osteoarthritis and rheumatoid arthritis (Rachow and Ryan

1985(a)). The lowest activity of NTPP and PPi concentrations are found in rheumatoid synovial fluid, perhaps due to the presence of increased proteolytic enzyme activity within the joint. The chondrocyte ecto-enzyme is membrane bound, whilst the synovial fluid enzyme appears to be predominantly soluble (Rachow and Ryan 1985(b)) suggesting solubilisation of NTPP from the chondrocyte. In a study of NTPP in human articular chondrocytes in monolayer culture the activity remained bound to the cell (Caswell and Russell 1985). However, in studies of porcine articular cartilage in organ and monolayer culture NTPP was released into the culture medium in a linear fashion over time (Rachow *et al* 1985, Rachow and McCarty 1986). Apart from species difference, the reason for such a discrepancy is unknown.

Rachow and Ryan (1985(b)), when characterising partially purified synovial fluid NTPP, discovered similarities between the synovial fluid and cartilage enzymes in that their divalent cation dependence, alkaline pH optimum and Kms were comparable. Despite these similarities, however, differences were noted in substrate specificity between the synovial fluid and cartilage enzyme. Whilst UTP was found to be the most active substrate for the cartilage enzyme, it was the least active for the synovial fluid enzyme. Synovial fluid NTPP also exhibited a much wider substrate specificity, showing in common with the liver plasma membrane enzyme an affinity for non-nucleoside triphosphate substrates. Such a discrepancy could be explained by the unpurified nature of the synovial fluid enzyme when studied, which makes the accurate characterisation of any enzyme difficult.

Articular cartilage NTPP still remains the favourable source for the synovial fluid enzyme. However, the process by which the enzyme is solubilised off the plasma membrane is unknown, as is the significance of NTPP in the synovial fluid. Although it is uncharacteristic for an ecto-enzyme to be shed from the plasma membrane of the cell (Manery and Dryden 1979), a mechanism may exist, facilitating a turnover of NTPP from the chondrocyte plasma membrane.

Although NTPP activity is reported as increased in pyrophosphate arthropathy cartilage, whether this is a localised abnormality in the diseased joint, or a generalised disturbance of articular cartilage PPi metabolism within an individual has not been investigated.

1.6 Articular cartilage and synovial fluid 5'nucleotidase activity.

5'nucleotidase (5NT) is another ecto-enzyme which resides on the exterior of the chondrocyte plasma membrane. 5NT catalyses the reaction:

$$AMP \rightarrow \text{Adenosine} + \text{phosphate}.$$

It has been postulated that 5NT acts together with NTPP and ALP in the recovery of nucleotides leaked from cells, 5NT by removing the phosphate groups, and allowing passage through the plasma membrane (Tran-Thi *et al* 1981).

Tenenbaum and colleagues (1981) and Muniz *et al* (1984) reported elevated

activity of 5NT in extracts of chondrocalcinotic compared to osteoarthritic and normal cartilage. It was postulated that elevated activity of this enzyme, hydrolysing nucleoside monophosphates, may result in the enhanced catabolism of nucleoside triphosphates and subsequent production of excess PPi, thus promoting CPPD crystal deposition. 5NT activity was also found to be elevated in calcium crystal containing synovial fluids (Rachow *et al* 1988(a), Wortmann *et al* 1991), compared to non-crystal containing osteoarthritic and rheumatoid synovial fluid, the 5NT presumably leaked from the articular cartilage. Consequently a specific association between elevated synovial fluid 5NT and articular calcium crystal deposition was proposed.

The source of synovial fluid 5NT has not been identified. However, as increased activity has been found in extracts of chondrocalcinotic cartilage this would seem a favourable source. In rheumatoid arthritis, synovial lining cells have been implicated as the source of synovial fluid 5NT (Farr *et al* 1973, Henderson *et al* 1980) the increased 5NT activity correlating with disease activity.

1.7 Articular cartilage and synovial fluid alkaline phosphatase activity.

In addition to increased production of PPi by articular cartilage, a defect in PPi hydrolysis may also be a factor leading to an excess of this ion, favouring CPPD deposition. An analogy supporting this hypothesis is the common occurrence of

CPPD deposition found in the rare congenital disorder hypophosphatasia in which a deficiency of alkaline phosphatase (ALP) results in increased plasma and urinary PPi concentrations.

ALP, an ecto-enzyme and a known pyrophosphatase (Cox *et al* 1967, Moss 1969) is thought to be responsible for hydrolysis of as much as 80% of PPi in the extracellular compartment (McGuire *et al* 1980(a)). Removal of extracellular PPi is therefore heavily reliant on the activity of this enzyme, itself responsive to a variety of physiological agents such as pH and substrate concentration. The pH optimum of the PPi hydrolytic activity of ALP decreases with decreasing PPi concentrations, such that at physiological concentrations of PPi the pH approaches 7.0 (Caswell *et al* 1983). Magnesium ions are also important in the regulation of ALP activity, optimal activity occurring when Mg^{++}/PPi is at a 1:1 ratio.

Activity of ALP has been reported to be depressed in extracts of chondrocalcinotic compared to osteoarthritic cartilage (Howell *et al* 1976, Tenenbaum *et al* 1981). Early studies measuring ALP and pyrophosphohydrolase activity in the synovial fluid of patients with pyrophosphate arthropathy also found decreased activity compared to synovial fluid from other arthropathies (Russell *et al* 1970, Yaron *et al* 1970, Good and Starkweather 1969). Subsequent studies, however, have failed to show a deficiency in ALP (McCarty *et al* 1971, Altman *et al* 1973(a), Giblisco *et al* 1985), acid phosphatase (Jacobelli *et al* 1978) or neutral inorganic pyrophosphatase activity (Rachow and Ryan 1985(a)) in pyrophosphate arthropathy

synovial fluid. However, normal synovial fluid ALP activity was not included as a control group in any previous studies, allowing a comparison only between disease states.

Evidence suggests that PPi hydrolysis in articular cartilage is not at fault at least in idiopathic chondrocalcinosis. Such a defect in PPi hydrolysis, however, would seem the favourable explanation for the association between chondrocalcinosis and hypophosphatasia, in which ALP activity is deficient.

Several divalent cations affect ALP activity, in particular magnesium, which as already mentioned exerts a complex regulatory effect (Caswell *et al* 1983). The effect of magnesium and other metal cations on ALP activity may account for the association between chondrocalcinosis and hyperparathyroidism, haemochromatosis and hypomagnesaemia, where calcium, iron and magnesium levels may exert an inhibitory effect on pyrophosphatase activity (McCarty *et al* 1970, Millazo *et al* 1981).

1.8 Metabolic diseases predisposing to calcium pyrophosphate dihydrate crystal deposition.

Several metabolic diseases have been associated with CPPD crystal deposition (McCarty *et al* 1974, Alexander *et al* 1982, Jones *et al* 1992) the best examples being the rarer conditions which often associate with premature widespread CPPD deposition. These include: hypomagnesaemia, haemochromatosis, hypophosphatasia and hyperparathyroidism. The physico-chemical mechanisms predisposing to crystal deposition in such a variety of metabolic diseases remain unclear, though differing putative effects on PPi metabolism are implicated, as illustrated in figure 3. In addition promotion of crystal nucleation by changes in the articular cartilage matrix (Russell 1976), inhibition of crystal dissolution (Russell 1976, Cheng and Pritzker 1981, Hearn and Russell 1978) and an increase in calcium (Russell 1976) have been postulated.

In vitro work supporting mechanisms of PPi metabolism have been confined to plasma and urinary measurements in metabolic disease. Since CPPD deposition is recognised as a localised intra-articular problem, studies within the joint on cartilage or synovial fluid PPi production and levels would therefore be of greatest interest.

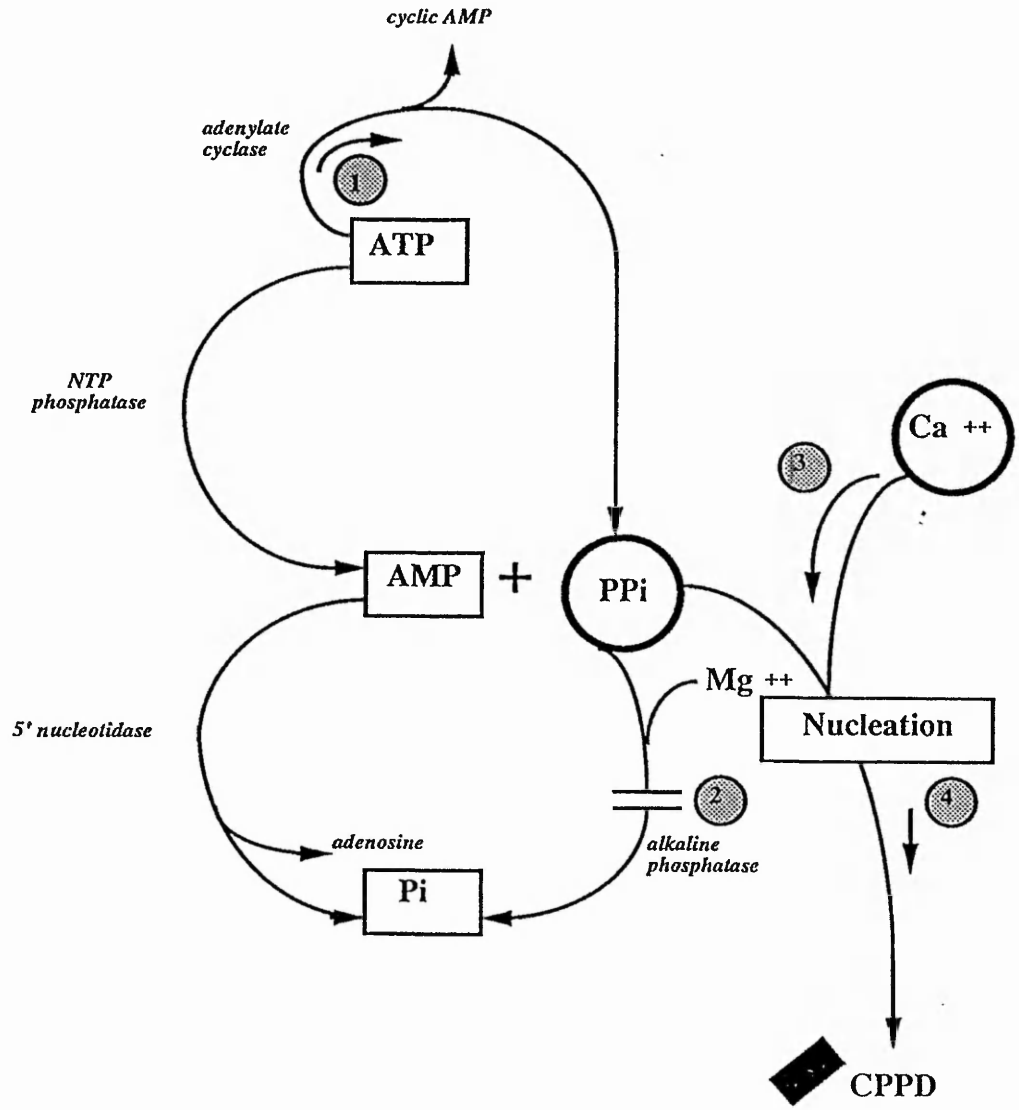


Figure 3: Putative effects on PPi metabolism by metabolic disease.

1. Increased adenylate cyclase activity: hyperparathyroidism.
2. Inhibition of alkaline phosphatase activity: hyperparathyroidism, haemochromatosis, hypophosphatasia, hypomagnesaemia.
3. Increased ionic calcium: hyperparathyroidism.
4. Promotion of crystal nucleation: haemochromatosis.

PPi = inorganic pyrophosphate; Pi = inorganic orthophosphate; AMP and ATP = adenosine mono- and triphosphate respectively.

1.8.1 Hypophosphatasia.

Hypophosphatasia is a rare hereditary disorder characterised by a deficiency of serum and tissue alkaline phosphatase (ALP), raised urinary phosphoethanolamine, and increased plasma and urinary PPi levels (Russell 1965, Sorensen *et al* 1978). As ALP is the principle pyrophosphatase hydrolysing PPi to orthophosphate, it is thought that the increased plasma and urinary PPi concentrations found in hypophosphatasia are due to a deficiency of this enzyme (Russell 1976, Fernley and Walker 1967). Increased PPi levels are known to inhibit apatite crystallisation (Fleisch and Bisaz 1962, Seigel *et al* 1983) resulting in poor bone mineralisation and a tendency to fracture in hypophosphatasia. At the same time, however, CPPD crystallisation may be promoted by an increase in the [PPi x Ca] ion product, predisposing the same subjects to pyrophosphate arthropathy (Chuck *et al* 1989).

The association between hypophosphatasia and CPPD crystal deposition is well documented (O'Duffy 1970, Eade *et al* 1981). The association is particularly supported by the occurrence of CPPD crystal deposition in juveniles with hypophosphatasia (Eade *et al* 1981). Studies on PPi metabolism have been limited due to the rarity of this disorder, and mechanisms leading to CPPD deposition remain uncertain. However, the disturbance in PPi metabolism found in hypophosphatasia accords with the favourable hypothesis of increased PPi levels leading to subsequent CPPD crystal deposition suggested in idiopathic chondrocalcinosis.

1.8.2 Haemochromatosis.

An association between haemochromatosis, a disorder in which there is excessive absorption and storage of iron, and CPPD crystal deposition has been demonstrated in several reports (Schumacher 1964, Atkins *et al* 1970, Dymock *et al* 1970, Angevine and Jacox 1974). In one study 41% of patients with haemochromatosis had CPPD crystal deposition, suggesting that the association is more than a chance occurrence (Hamilton 1968). A specific arthropathy has been reported in a high proportion of patients with haemochromatosis (Hamilton 1968, Dymock *et al* 1970) and the degree of chondrocalcinosis has been shown to correlate with the severity of this arthropathy (Atkins *et al* 1970). It is possible, therefore, that CPPD deposition may occur as a result of pre-existing joint damage, rather than in association with the metabolic disorder.

Other mechanisms suggested for the promotion of CPPD crystal formation in haemochromatosis include effects on PPi metabolism and promotion of crystal nucleation by iron salts. In terms of PPi metabolism, Fe^{++} may be effective in increasing PPi concentrations by inhibiting pyrophosphatase activity. McCarty *et al* (1970) demonstrated that erythrocyte intracellular pyrophosphatase is inhibited by ferrous ions. Iron salts may also act by nucleating for CPPD crystals. Hearn *et al* (1978) demonstrated that Fe^{+++} promoted crystal deposition and lowers the ion formation product, [Ca x PPi], necessary for crystal formation *in vitro*. In spite of such evidence, however, iron salts are not particularly evident at sites of

CPPD deposition in haemochromatosis and the removal of iron by venesection fails to prevent the development of chondrocalcinosis (Hamilton 1981).

1.8.3 Hyperparathyroidism.

Several previous reports have associated hyperparathyroidism, an endocrine disorder leading to increased serum calcium concentrations, with CPPD crystal deposition (Alexander *et al* 1982, Dodds *et al* 1968, Grahame *et al* 1971, Zvaifler 1962). The reported incidence of chondrocalcinosis associated with hyperparathyroidism has varied from 18% (Dodds *et al* 1968) to 40% (Glass and Grahame 1976).

Various postulates have been given to explain this association, mainly involving the effects of increased systemic calcium levels. Increased calcium alone may promote CPPD crystal deposition in the joint by increasing the ionic product. In addition pyrophosphatase activity is inhibited by calcium ions (McCarty *et al* 1970) which would in turn cause an increase in PPi. A systemic increase in PPi has not been consistently demonstrated in hyperparathyroidism. Although elevated urinary PPi levels were found by Avioli *et al* (1965, 1966) subsequent studies failed to confirm increased urinary or plasma levels (Lewis 1966, Russell *et al* 1971).

Previous studies have suggested that a disturbance in PPi metabolism via NTPP

activity is not responsible for promoting CPPD deposition in hyperparathyroidism. Caswell and Russell (1988) demonstrated that increasing calcium concentrations in chondrocyte monolayers did not have a significant effect on increasing the activity of NTPP and PPi elaboration. Ryan *et al* (1989) found no increase in the accumulation of PPi in chondrocyte culture media either from adding porcine parathyroid hormone or by varying the ambient calcium ion concentration. However, the long term effects of increased calcium and parathyroid hormone on chondrocytes *in vivo* are difficult to determine *in vitro*.

A more indirect relationship between increased parathyroid hormone and crystal deposition may exist. Parathyroid hormone has been shown to have a mitogenic effect on chondrocytes in the presence of calcium ions and increases adenylate cyclase activity in the plasma membrane (Schluter *et al* 1989, Centralla *et al* 1989). This increased adenylate cyclase activity may result in increased local PPi (McGuire *et al* 1980(a), Tell *et al* 1973). However, whether this PPi is leaked to the extra-cellular space is unknown. Parathyroid hormone also increases cAMP and glycosaminoglycan synthesis by chondrocytes (Enomoto *et al* 1989). Thus, increased extracellular PPi could result from exportation via an increase in matrix biosynthesis.

1.8.4 Hypomagnesaemia.

Hypomagnesaemia, due to a rare inherited renal tubular disorder, has been associated with CPPD crystal deposition on several occasions (Runeberg *et al* 1975, Resnick and Rausch 1974, Milazzo *et al* 1981). The association would seem to be a true one due to the high prevalence of CPPD crystal deposition in young adults affected by this uncommon condition.

A deficiency in magnesium ions could potentially lead to multiple metabolic problems, magnesium being a co-factor for many enzymatic reactions including the hydrolysis of PPi by alkaline phosphatase and other pyrophosphatases. As magnesium ions exert a complex regulatory effect on ALP, a disorder in the clearance of PPi in hypomagnesaemia, leading to CPPD crystal deposition may be a possible mechanism involved in the association.

Although NTPP activity has previously been found to be magnesium dependent (Bischoff 1975), a reduction in magnesium was not found to effect the activity of chondrocyte NTPP *in vitro* (Caswell and Russell 1984). On this evidence, effects on PPi metabolism via NTPP activity in hypomagnesaemia would seem not to contribute to the promotion of CPPD crystal deposition.

1.8.5 Hypothyroidism.

An association between hypothyroidism and chondrocalcinosis was first reported by Dorwart and Schumacher (1975), who found chondrocalcinosis in seven out of eleven patients with hypothyroidism. Since this first report there has been conflicting evidence for and against an association between these two age-related disorders. Alexander *et al* (1982) found an increased incidence of hypothyroidism in CPPD patients, whilst other authors (McCarty 1974, Komatireddy 1989, Smith 1990, Job-Deslandre *et al* 1993) found no increased incidence of hypothyroidism. A recent study by Jones *et al* (1992) reported a small but significant association between hypothyroidism and chondrocalcinosis by performing meta-analysis on data from two separate studies. Studies on possible mechanisms relating hypothyroidism to CPPD deposition are scarce and no previous reports on PPi metabolism in hypothyroidism are evident. Synovial cells and chondrocytes are known to express a thyroid stimulating hormone responsive adenylate cyclase (Newcombe *et al* 1972, Corvol 1972), which may be involved in the pathogenesis of myxoedema arthritis. As PPi is a by-product of cAMP production, increased activity of adenylate cyclase via stimulation by thyroid stimulating hormone could potentially lead to increased local PPi levels. This is dependent, however, on PPi reaching the extracellular space.

1.8.6 Other associated metabolic disorders.

Several other metabolic disorders have also been associated with CPPD crystal deposition. However, due to either lack of data or the rarity of the specific condition, it remains uncertain whether these associations are true ones. CPPD crystal deposition has been found in Wilson's disease (Feller and Schumacher 1972, Golding and Walshe 1977). In the former report occurring in two patients aged twenty-six and thirty-one. It would seem that there is a true association here due to the uncommon presence of CPPD crystal deposits at such a young age. It has been suggested that copper ions, by inhibiting pyrophosphatases, may promote CPPD deposition in this rare disease (McCarty *et al* 1970). An association has also been reported with ochronosis (Bywaters *et al* 1970, Reginato *et al* 1973, Rynes *et al* 1975), it being postulated that the cartilage pigmentation present in ochronosis may facilitate crystal deposition.

Associations between Paget's disease of bone (Radi *et al* 1970) diabetes mellitus, hypertension, and artherosclerosis (McCarty 1972, Hamilton 1976) have also been cited. However, it is questionable whether any of these conditions represent more than a chance concurrence of two common age-related diseases, as controlled studies have suggested (Boussina *et al* 1971, McCarty *et al* 1974, Boussina *et al* 1976, Hamilton 1976, Alexander *et al* 1982).

1.9 Calcium pyrophosphate dihydrate crystal deposition and associated joint diseases.

1.9.1 Osteoarthritis.

An association between CPPD crystal deposition and joint damage has been implicated due to the frequent finding of chondrocalcinosis with co-existing joint disease. The most common association reported is with osteoarthritis where CPPD crystal deposition has been found to exist in 40-70% of osteoarthritic patients (Hamilton 1976). Several other studies have also demonstrated this positive correlation between chondrocalcinosis and osteoarthritis (Sokoloff and Varma 1988, Felson *et al* 1989).

Although osteoarthritis and chondrocalcinosis commonly co-exist, the relationship between these two conditions is complicated by their frequent existence in the absence of one another, and by their strong relationship with ageing, though Felson *et al* (1988), have shown that chondrocalcinosis is associated with osteoarthritis independent of age. Dieppe *et al* (1982) proposed that osteoarthritis is a pre-disposing factor to chondrocalcinosis, as their study suggested a progression from osteoarthritis to more widespread disease associated with pyrophosphate arthropathy. This finding of joint damage promoting crystal deposition is further strengthened by the additional reports of chondrocalcinosis following meniscectomy (Doherty *et al* 1982), and localised pyrophosphate arthropathy as

a late complication of juvenile chronic arthritis (Doherty and Dieppe 1984), joint instability (Settas *et al* 1982) and trauma. An amplification loop hypothesis was proposed by Dieppe *et al* (1982) to explain the frequent association of CPPD deposition with pre-existing joint disease. They hypothesised that joint damage leads to crystal deposition and in addition further crystal shedding which in turn perpetuates joint damage and disease.

Although mechanisms behind the association between joint disease and chondrocalcinosis are uncertain, CPPD crystal deposition as a secondary event to osteoarthritis would agree with the present knowledge of PPi metabolism in that the damaged or synthetically active chondrocytes present in osteoarthritic cartilage may leak nucleoside triphosphates, leading to a subsequent increase in local PPi levels promoting crystal deposition. The increased synovial fluid PPi levels found in osteoarthritis may reflect this process (Silcox and McCarty 1974).

Other possible factors produced by joint damage may be the loss of crystal inhibitors, for example proteoglycan aggregates which inhibit crystal formation and are known to be decreased in the osteoarthritic joint (Altman *et al* 1973(b)), or the presence of promoters of crystal nucleation for example epitaxy on apatite crystals which are commonly found in osteoarthritis (Dieppe and Calvert 1983).

1.9.2 Rheumatoid arthritis.

Studies recording the incidence of rheumatoid arthritis and CPPD crystal deposition have reported conflicting findings. A number of uncontrolled studies have suggested an association between rheumatoid arthritis and CPPD deposition (Good and Rapp 1967, Moskowitz and Garcia 1973, Bywaters 1972, Rubenstein, McCarty, Resnick *et al* 1980). In subsequent controlled studies an association was not found between rheumatoid arthritis and CPPD deposition (Good and Rapp 1969, Hollingworth *et al* 1982), although the control groups in these two studies may have been inappropriate. The study by Good and Rapp (1969) used gouty patients as the control group, which may be inappropriate as an association between gout and CPPD has since been reported. The control group in the study by Hollingworth had an average age of fifty-five, which is low for chondrocalcinosis and may therefore have hidden the negative association between rheumatoid arthritis and CPPD deposition found by two subsequent, more carefully controlled studies (Doherty *et al* 1984, Brasseur *et al* 1987). In addition to the radiographic study comparing rheumatoids to normals, Doherty *et al* (1984) found the same negative association with synovial fluid CPPD crystal presence in rheumatoid compared to osteoarthritic synovial fluid. A finding later confirmed by Hamilton *et al* (1990).

Despite the presence of joint damage in rheumatoid arthritis, CPPD crystal deposition does not seem to be promoted in this disease unless accompanied by

atypical radiographic features more akin to the hypertrophic response found in osteoarthritis (Doherty *et al* 1984). This agrees with the putative association of CPPD crystal deposition and hypertrophic (osteophyte, cyst, remodelling) rather than atrophic joint disease (Doherty and Dieppe 1988). One explanation may be that the more extensive destruction of cartilage in rheumatoid arthritis, on which CPPD crystals rely for deposition, decreases the risk of chondrocalcinosis. Synovial fluid PPi levels in rheumatoid arthritis have been reported as low (Silcox and McCarty 1974, Pattrick *et al* 1991). This is perhaps due to either the presence of increased pyrophosphatase activity, or increased removal from the joint. It has also been suggested that the rheumatoid inflammatory process may promote crystal dissolution (Zyskowski 1983).

1.9.3 Gout.

An association between CPPD crystal deposition and gout has been established for some time. Currey *et al* (1966) found that out of 34 patients with chondrocalcinosis 8 had gout. McCarty (1972) also reported a 5 per cent incidence of hyperuricaemia and a 34 per cent incidence of gout with chondrocalcinosis. The increased association with urate gout, rather than with hyperuricaemia, was also reported in a controlled study by Stockman *et al* (1980). This association may be representative of one crystal type nucleating another (epitaxy); a mechanism which may exist in the association between chondrocalcinosis and gout. In the same

study, gouty patients with chondrocalcinosis had higher radiological scores for osteoarthritis and longer duration of symptoms. Thus implicating joint damage in gout as the mechanism promoting CPPD crystal deposition.

PPi metabolism in the gouty joint has been studied in terms of synovial fluid PPi levels. These were found to be increased in comparison to rheumatoid synovial fluid, but not elevated above osteoarthritic or pyrophosphate arthropathy levels (Altman *et al* 1973(a)).

1.9.4 Other joint diseases associated with calcium pyrophosphate dihydrate crystal deposition.

Articular amyloid deposition and chondrocalcinosis have been associated in several reports (Kaplinski 1976, Teglbjaerg *et al* 1979, Egan *et al* 1980). Amyloid arthropathy and chondrocalcinosis have been associated by Ryan and colleagues (1982), who found that 3 patients with symptomatic articular amyloidosis also had chondrocalcinosis. Amyloid deposition at non-articular sites has also been associated with calcification, mainly in the form of hydroxyapatite, suggesting that amyloid may act as a promoter of calcification. Amyloid fibres have been shown to enhance glycosaminoglycan synthesis by fibroblasts which could result in a concomitant increase in PPi concentration, produced as a by-product. Amyloid deposits may also favour crystal formation by binding PPi, as they bind

diphosphonate analogues of PPi (Kula *et al* 1977), and calcium (Yood *et al* 1981).

Hypermobility and chondrocalcinosis were associated initially by Bird *et al* (1978), who found four out of sixteen patients with generalised hypermobility had chondrocalcinosis. It was proposed that mechanical factors resulting from joint laxity in hypermobility may promote CPPD crystal deposition. This proposed mechanism is supported by the finding of chondrocalcinosis in association with unstable joints (Settas *et al* 1982) and neuropathic joints (Jacobelli *et al* 1973), where CPPD crystal deposition may again be secondary to pre-existing joint damage.

1.10 Familial Pyrophosphate Arthropathy.

In addition to the metabolic disease-associated and sporadic forms of CPPD crystal deposition, an hereditary predisposition has also been identified. Since Sitaj and Zitnan (1957) initially described hereditary chondrocalcinosis several pedigrees from various countries have been described (Zitnan and Sitaj 1963, Moskowitz and Katz 1964, Reginato *et al* 1970, Van de Korst *et al* 1974, Gaucher *et al* 1977, Rodriguez-Valverde *et al* 1980, Gaudreau *et al* 1981, Bjelle 1981, Sakaguchi *et al* 1982, Richardson *et al* 1983, Fernandex Dapica and Gomez-Reino 1986, Doherty *et al* 1991(b)).

Different modes of inheritance have been shown for these different pedigrees, although autosomal dominant inheritance seems the most common. The recognition of the majority of familial cases has come about by their presentation at an early age with severe polyarticular disease, often with atypical spinal involvement. The most severe cases have been reported from Chile and Czechoslovakia with disease onset before twenty years of age; tissue typing in these cases revealed homozygotes. However, in the Spanish series and in heterozygotes from the Czech and Chilean series, a less dramatic disease has been identified. Late onset symptoms, predominance in women, mild clinical disease and oligoarticular chondrocalcinosis make these familial cases indistinguishable from the sporadic form (Rodriguez-Valverde *et al* 1980, Balsa *et al* 1990). Thus it may be that the familial form of the disease is much more common than previously thought,

perhaps even influencing the sporadic forms of pyrophosphate arthropathy.

The mechanisms of familial predisposition remains uncertain, though both a metabolic disturbance of cartilage matrix (Bjelle 1981) and a generalized abnormality of PPi metabolism have been implicated (Lust *et al* 1981, Ryan *et al* 1986). In the former, a morphological and biochemical study found that hydroxyproline levels were low and keratin sulphate levels were elevated in familial cartilage compared to normal controls, and that such an abnormality could promote CPPD crystal deposition.

In addition an abnormality of PPi metabolism has been found in non-articular tissue in familial pyrophosphate arthropathy patients by two separate groups. Lust *et al* (1981), when studying a French kindred, found elevated intracellular PPi levels in cultured skin fibroblasts and Epstein-Barr virus transformed lymphoblasts from affected family members compared to non-affected members and unrelated normal controls. Subsequent studies by Ryan *et al* (1986) confirmed elevated PPi levels in skin fibroblasts of patients with familial pyrophosphate arthropathy. However, they also found increased PPi in fibroblasts from patients with sporadic pyrophosphate arthropathy, suggesting that a similar abnormality of PPi metabolism may also be responsible for non-familial disease. Both studies were hampered, however, by the considerable overlap found between normal and familial intracellular PPi levels. The mechanism for increased intracellular PPi levels in these cell types is uncertain. Lust *et al* (1981) found no excretion or

external accumulation of PPi from lymphoblasts or fibroblasts cultures, unlike findings in chondrocyte cultures where the extra-cellular build up of PPi is thought to contribute to crystal deposition. Ryan *et al* (1986) also found elevated ecto-NTPP activity on skin-derived fibroblasts from patients with sporadic CPPD deposition which correlated with intracellular PPi levels, suggesting that intracellular PPi may be generated by this enzyme before its translocation. Thus two possible biochemical markers in patients with familial and sporadic pyrophosphate arthropathy have been identified, suggesting a generalised abnormality of PPi metabolism expressed in non-articular tissues.

Previously, studies on PPi metabolism in familial pyrophosphate arthropathy have focused on non-articular tissues, in search of a systemic abnormality. It is of interest, therefore, how chondrocyte extracellular PPi metabolism is affected by this familial disease, and whether ecto-NTPP activity may be increased in the articular cartilage of familials. Synovial fluid studies, previously lacking, are therefore of great interest.

1.11 Project aims and objectives.

From the introductory literature it is evident that age, metabolic disease and familial tendency all predispose to articular CPPD crystal deposition. The effects of these predisposing factors on articular PPi metabolism have not, however, been investigated.

The aims of this study are, therefore, to determine the effects of conditions that predispose to CPPD crystal deposition and effects of disease activity or clinical inflammation on articular PPi metabolism. In addition, to determine whether a general defect of articular PPi metabolism is evident in sporadic pyrophosphate arthropathy.

The specific objectives of the study are: (a) To measure synovial fluid PPi concentrations and synovial fluid NTPP activity in subjects with various arthritic diseases, underlying metabolic disorders, and in subjects with familial predisposition. (b) To investigate the effects of ageing on articular PPi metabolism in a large cohort of normal subjects. (c) To assess the effects of disease activity on articular PPi metabolism; the activities of synovial fluid NTPP, 5NT and ALP and PPi concentrations will be studied from arthritic joints assessed according to their clinical inflammatory state preceding aspiration. (d) To determine whether there is a generalised alteration of chondrocyte PPi metabolism in sporadic pyrophosphate arthropathy; NTPP activity and PPi elaboration will be compared

in chondrocyte cultures from involved and uninvolved knees from the same subject.

CHAPTER 2: Subjects.

2.1 Arthritic diagnostic groups.

Synovial fluid for study was obtained from the knees of patients attending the Rheumatology Unit, Nottingham City Hospital. The knee was chosen since it is the commonest site for CPPD deposition and chondrocalcinosis, and is a large joint that is readily accessible for aspiration. The major diagnostic groups studied were pyrophosphate arthropathy, osteoarthritis and rheumatoid arthritis, diagnosed according to the criteria below. Arthritic groups were selected to be late middle-aged or elderly as CPPD crystal deposition is uncommon below the age of 55.

2.1.1 Pyrophosphate arthropathy.

Sixty-one patients with pyrophosphate arthropathy were studied (40 female, 21 male; mean age 77), defined as a persistent (> 3 months) symptomatic arthropathy with synovial fluid CPPD crystals and radiographic features of osteoarthritis, with or without chondrocalcinosis.

2.1.2 Osteoarthritis.

Fifty-nine patients with osteoarthritis (29 female, 30 male, mean age 70) were included in the study. These subjects had symptomatic seronegative arthropathy with radiographs showing cartilage loss plus subchondral sclerosis or osteophytes, or both. None had radiographic chondrocalcinosis, synovial fluid CPPD crystals or evidence of other primary joint disease.

2.1.3 Rheumatoid arthritis.

Forty-four patients (29 female, 15 male, mean age 63) with rheumatoid arthritis were included in the study. These subjects fulfilled the American Rheumatism Association criteria for classic or definite disease (A Committee of the ARA 1958).

2.2 Assessment of clinical inflammation.

Synovial fluid samples from the above disease categories were further assessed as "active" or "inactive" according to clinical inflammation at the time of aspiration. The global assessment of inflammation was made using a summated score of six clinical variables as described in table 2. A score of 0-2 was regarded

	Inactive	Active
Individual Parameters		
Increased warmth	Absent	Present
Effusion	Absent-mild/not tense	Moderate-marked/tense
Synoviai Thickening	Absent	Present
Joint line tenderness (0 - 3)	0 - 1	2 - 3
Early morning stiffness	<1 hour	1 hour or more
Inactivity stiffness	<15 minutes	15 minutes or more
Global assessment	<3 parameters	4 - 6 parameters

Table 2: Knee assessment for clinical inflammation

as "inactive", whilst a score of 4-6 was considered "active" (Doherty *et al* 1988). Intermediate joints scoring 3 were not included.

2.3 Metabolic disease groups.

2.3.1 Primary hyperparathyroidism.

Twenty-one subjects with primary hyperparathyroidism were studied (12 women and 9 men, with a mean age of 72, age range 58-80). All had concurrent hypercalcaemia (albumin corrected) and elevated serum parathyroid hormone levels (ie > 80 ngl^{-1}: normal 10-55 ngl^{-1}, measuring intact molecule by immuno-radiometric assay).

2.3.2 Idiopathic haemochromatosis.

Five subjects with haemochromatosis were studied (3 men and 2 women, with a mean age of 63, age range 54-70). All had serum ferritin levels > 3000 μgl^{-1} (normal 20-300 μgl^{-1}). Liver biopsy was performed on 4 patients: in each case histological changes consistant with idiopathic haemochromatosis were demonstrated. 1 patient had associated diabetes, 2 were siblings with familial disease.

2.3.3 Hypomagnesaemia.

Two female subjects with hypomagnesaemia were studied, ages 42 and 46 with persistant hypomagnesaemia (mean serum levels 0.45 $mmoll^{-1}$, range 0.3-0.6 $mmoll^{-1}$: normal 0.7-1.0 $mmoll^{-1}$) due to a congenital isolated renal tubular defect. None had evidence of Bartter's syndrome.

2.3.4 Adult onset hypophosphatasia.

Five subjects with hypophosphatasia were studied (3 women and 2 men, with a mean age of 64, age range 38-85) with persistant low serum alkaline phosphatase activities (mean 50 IUl^{-1}, range 31-69 IUl^{-1}; normal 80-280 IUl^{-1}) and abnormal urinary excretion of phosphoethanolamine (mean 0.051, range 0.018-0.068 mmol/mmol creatinine; normal not detected). 2 patients presented with recurrent fractures and osteopenia, 2 with calcific periarthritis, and 1 was the asymptomatic father of one of the patients.

2.3.5 Primary hypothyroidism.

Twenty-seven subjects with hypothyroidism were studied (18 women and 9 men, with a mean age of 62, age range 42-78). All had biochemical evidence of hypothyroidism and marked elevation of serum thyroid stimulating hormone (ie. > 50 mUl^{-1}; normal 0.1-4.3 mUl^{-1}).

At the time of study all patients were untreated with respect to their metabolic disease and had investigative evidence of current metabolic abnormality. None had clinical or biochemical evidence of other co-existing metabolic disease (blood screening included calcium, alkaline phosphatase, ferritin, magnesium and thyroid function). None had symptomatic or clinical evidence of chronic pyrophosphate arthropathy. Plain radiographs of knees were obtained in all patients to determine presence of chondrocalcinosis and to exclude unsuspected structural arthropathy.

2.4 Familial pyrophosphate arthropathy subjects.

2.4.1 Family groups.

Five unrelated families were detected following presentation of index cases to Buxton (family 1), Telford (family 2) or Nottingham (families 3,4,5).

Available first degree relatives were questioned and examined in respect of locomotor and medical problems. Radiographs of knees and hands were undertaken in asymptomatic adult relatives to screen for chondrocalcinosis. In chondrocalcinotic, symptomatic or clinically arthropathic subjects, the radiographic screen also included the pelvis, shoulders and cervical, thoracic and lumber spine, plus other clinically involved sites. Routine metabolic screening of affected subjects included: full blood count, alkaline phosphatase, calcium, magnesium, ferritin, uric acid and thyroid function.

2.4.2 Sporadic controls.

Controls for the familial cases were obtained from fifty-nine patients (29 female, 30 male) with symptomatic sporadic pyrophosphate arthropathy (synovial fluid CPPD crystals ± radiographic chondrocalcinosis), having no evidence of predisposing metabolic disease and no suggestive family history (pyrophosphate

arthropathy and chondrocalcinosis involved no more than four joint sites in any patient, and were confined to one or both knees in 37).

2.5 Normal subjects.

The normal control group consisted of fifty volunteers (29 female, 21 male, mean age 44, age range 26-63) with no clinical evidence of metabolic or joint disease. In those aged older than 50 years radiographs were obtained to exclude occult osteoarthritis or chondrocalcinosis. The normal group was comprised of medical undergraduates, hospital and laboratory staff, spouses of rheumatology patients and members of the Women's Royal Voluntary Service.

CHAPTER 3: Methods.

3.1 Sample collection.

3.1.1 Synovial fluid.

Synovial fluid for study was collected into either 5ml or 20ml sterile containers immediately after aspiration. A sample of synovial fluid was examined fresh for the presence of CPPD crystals and other calcium containing particles for diagnostic purposes. For PPi, NTPP, protein, ALP and 5NT assays, samples were spun at 2500g for 15 minutes at 4°C to remove cells and crystals and the resulting supernatant was aliquoted and stored at -80° C until required.

3.1.2 Urine.

Second void fasting morning urines were collected into sterile containers and frozen at -80°C until required.

3.2 Identification of synovial fluid calcium pyrophosphate dihydrate crystals.

Synovial fluid CPPD crystals were identified in unspun fresh synovial fluid by their characteristic rhomboid habit and sign of birefringence (Currey and Vernon-Roberts 1976); that is the difference between the refractive index (the speed at which plane polarised light passes through the crystal) measured with light parallel to the long axis and with light perpendicular to the long axis. The polarising microscope achieves this by filtering out all but one direction of vibrating light.

To determine the sign of birefringence, the crystal is orientated with its long axis at the 45° position that intersects the N-E angle. In this position the crystal exhibits straight extinction, that is, it appears maximally bright on a black background. If the quartz red plate is now inserted into the microscope tube, the interference colours of the crystal will be shown. The interference colour of the crystal depends upon whether the geometric long axis corresponds to the optically slow, or optically fast axis. The retardation of light induced by the red plate, will either be further or less retarded, this being reflected by a change in the interference colour. If the slow axis of the crystal is along its geometric long axis, the retardation of the light in the SW-NE position is increased. the resulting interference colour is blue. Such a crystal is positively birefringent. This is typical of CPPD crystals. If the crystal is then rotated to the NW-SE position, there will be a change of colour from blue to yellow. Due to the fast axis now being parallel to the slow axis of

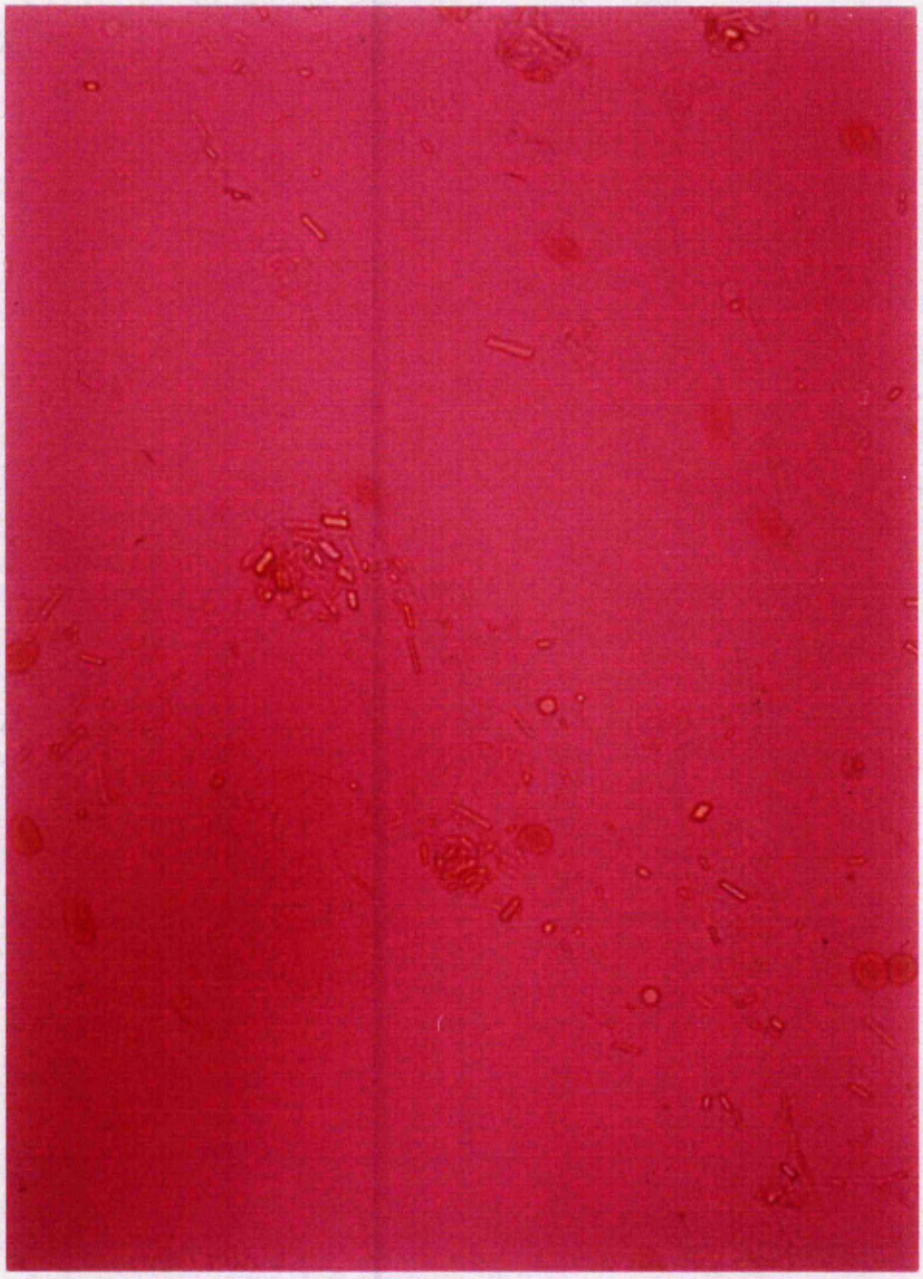


Figure 4: Synovial fluid calcium pyrophosphate dihydrate crystals under compensated polarised light (x400).

the red wedge.

If, however, the fast axis of the crystal is along the long axis of the crystal, the reverse occurs, such crystals show negative birefringence. this is typical of monosodium urate monohydrate.

Figure 4 shows an example of CPPD crystals in synovial fluid under compensated polarised light, exhibiting positive birefringence; the CPPD crystal appears blue when orientated in the SW-NE position and yellow when the crystal is rotated to the NW-SE position.

3.3 Assay of synovial fluid inorganic pyrophosphate.

Synovial fluid inorganic pyrophosphate (PPi) was measured by a modification of a specific, sensitive radiometric assay (McGuire *et al* 1980(b)) involving the conversion of PPi to labelled 6-phosphogluconate, as illustrated in figure 5. 200µl aliquots of synovial fluid were extracted with 100µl of trichloroacetic acid (TCA: 50% w/v) (BDH) and centrifuged at 2500g for 15 minutes at 4°C. The TCA was then removed by addition of 1 volume tri-n-octylamine (BDH) dissolved in 3 volumes 1,1,2-trichloro-fluoroethane (BDH). The resulting aqueous phase (500µl) was neutralised by the addition of 100µl of 0.1M tris /8mM magnesium acetate buffer (pH 8). The final incubation mix contained in a volume of 140µl: tris

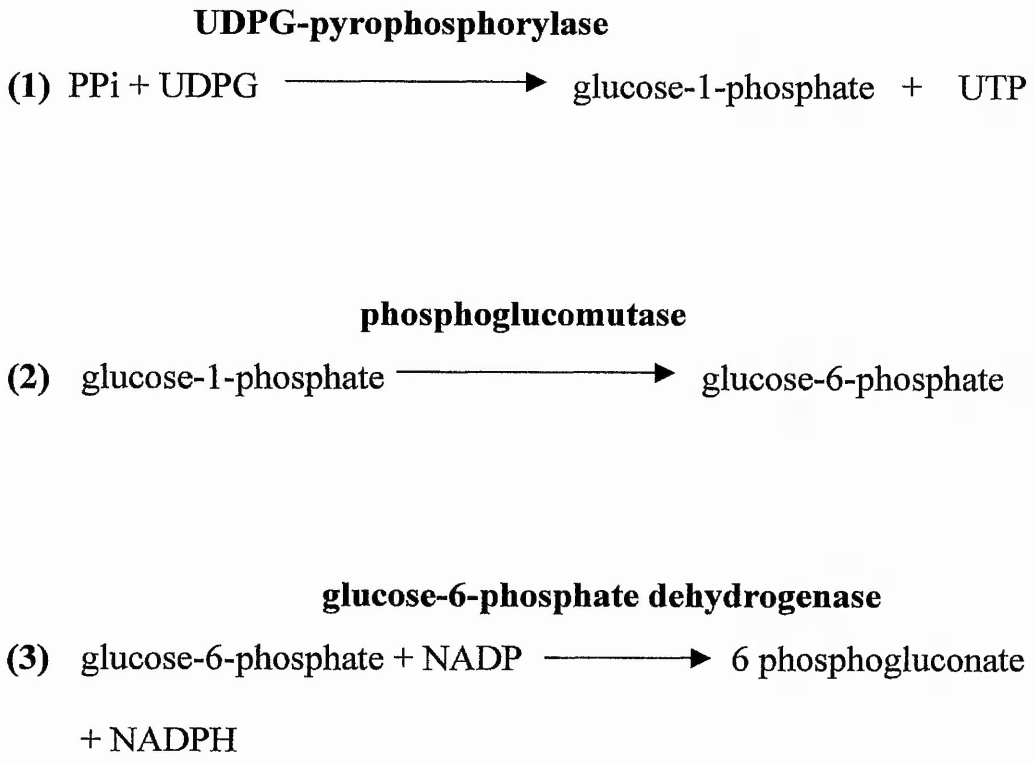


Figure 5: The triple reaction converting PPi to labelled 6-phosphogluconate.

(57mM) (Sigma); magnesium acetate (5.2mM) (Sigma); NADP (4μM) (Sigma); glucose 1,6-diphosphate (18.6μM) (Boehringer Mannheim (BM)); UDPG (7.5μM) (BM); glucose-6-phosphate dehydrogenase (0.4 units) (BM); phosphoglucomutase (0.2 Units) (BM); uridine diphosphoglucose pyrophosphorylase (0.136 Units) (BM); tritiated UDPG (0.8μCi; specific activity 3-10 Ci/mmol) (Amersham); and 40μl of sample or standard PPi (0.5-6μM) (Sigma).

The incubation reaction was performed in triplicate for 75 minutes at 37°C in a shaking waterbath, then terminated by cooling on ice. 250μl of a 2% activated charcoal (BDH) suspension (de-ionised water) was added to separate any unreacted substrate from labelled 6-phosphogluconate. After mixing, samples were centrifuged for 15 minutes at 2500g at 4°C. 200μl of supernatant was then counted for radioactivity in 4ml scintillation fluid.

To measure for any hydrolysis of PPi to orthophosphate (Pi) during incubation and extraction, tracer ^{32}P-PPi (Dupont) added to the initial sample was measured. Pi was removed by mixing 100μl of each sample with an equal volume of ammonium molybdate (5%w/v) (BDH) in HCL (2.7M) (BDH). The resulting phosphomolybdate complex was then recovered into isobutanol/light petroleum (4:1 v/v) (BDH). The aqueous phase was then counted for remaining ^{32}P-PPi in 0.1M HCL.

3.4 Assay of urine inorganic pyrophosphate.

Estimation of urinary PPi was the same as for synovial fluid. Results were corrected for creatinine and expressed as μM PPi/mM creatinine.

3.5 Assay of synovial fluid nucleoside triphosphate pyrophosphatase activity.

Synovial fluid NTPP activity was expressed as PPi generated in the presence of added ATP. 200μl aliquots of synovial fluid were incubated at 37°C in the presence of 120μM ATP (Sigma) and tracer ^{32}P-PPi. The incubation mixture contained: 5.4mM KCl; 0.8mM $MgSO_4$; 1.8mM CaCl; 0.118mM NaCl; 1mM NaH_2PO_4; 5.56mM glucose; and 20mM Hepes (all Sigma). After 30 minutes in a shaking waterbath, incubation was terminated by cooling on ice and samples were extracted with TCA and PPi generated was measured as above. NTPP activity is expressed as μmol PPi/minute/mg total protein.

3.6 Assay of synovial fluid alkaline phosphatase activity.

Synovial fluid alkaline phosphatase (ALP) was measured using a reagent kit (BDH). The kit measures ALP activity by the rate of dissociation of p-nitrophenylphosphate to p-nitrophenolate and phosphate. The synovial fluid

being tested for ALP activity was reacted in a cuvette with the substrate solution which contained: 10mM p-nitrophenylphosphate; 0.5mM $MgCl_2$; 1M diethanolamine HCL buffer, pH 9.8. The absorbance was recorded over 3 minutes at 25°C, in a spectrophotometer. The mean absorbance difference was then calculated per minute and substituted into the formula:

volume activity U/l = mean absorbance difference/min x 5460.

3.7 Assay of synovial fluid 5'nucleotidase activity.

Synovial fluid 5'nucleotidase activity was measured by a diagnostic kit (Sigma). The procedure in the kit employs an enzyme kinetic method reported by Arkesteijn (1976); the hydrolysis of adenosine monophosphate by 5'nucleotidase yields adenosine and inorganic phosphate. Adenosine deaminase then deaminates the adenosine, producing inosine and ammonium ion. The ammonium ion reacts with 2-oxoglutarate in the presence of reduced NADH to form glutamate and NAD. The rate of NAD formation produces a decrease in absorbance at 340nm and is directly proportional to the rate of adenosine formation and, hence 5'nucleotidase activity.

The Assay reagent which was prepared by the addition of deionised water contains in a volume of ml: AMP 3.2mM, NADH 0.2mM, 2-Oxoglutarate 3.7 mM, GLDH 11,000 U/L, ADA 400 U/L, B-Glycerophosphate. The procedure was as follows: in duplicate using deionised water as a blank: 1.5ml assay reagent was pipetted

into a cuvette then 100µl synovial fluid was added and mixed by inversion. The cuvette was allowed to stand for 5 minutes and then the absorbance was read at 340nm in a spectrophotometer, exactly 5 minutes later the absorbance was read again. The initial absorbance was subtracted from the final absorbance and multiplied x 515 to give 5'nucleotidase activity in U/L.

3.8 Assay of synovial fluid total protein.

Synovial fluid total protein was measured by the Biuret method. 2.5ml Biuret reagent (Sigma) was added to 50µl synovial fluid, protein standard or distilled water (blank) in duplicate, mixed thoroughly in a cuvette and allowed to stand for 15 minutes at room temperature. The absorbancies were then read a 540nm in a spectrophotometer. The total protein is calculated by the following equation:

absorbance of sample / absorbance of standard x 8 (g/dl^{-1}) (concentration of standard), giving a synovial fluid total protein result in g/dl^{-1}.

3.9 Assay of urinary creatinine.

Urinary creatinine was measured by a quantitative, colourometric diagnostic kit (Sigma). In this method, colour derived from creatinine is destroyed at acid pH. The difference in colour intensity measured at or near 500nm before and after

acidification is proportional to the sample creatinine concentration. The procedure was as follows in duplicate: 3mls alkaline picrate solution (containing: picric acid, sodium borate, surfactant and sodium hydroxide) was added to cuvettes containing: (a) 300µl distilled water (blank); (b) 300µl standard creatinine; and (c) 300µl sample (urine diluted 1 in 10 in distilled water). After standing at room temperature for 8-12 minutes the initial absorbance was read at 500nm. 100µl acid reagent (containing a mixture of sulphuric acid and acetic acid) was then added to (a), (b) and (c) and allowed to stand for 5 minutes at room temperature. The final absorbances were then read at 500nm. Sample creatinine was then calculated by subtracting the final absorbance from the initial absorbance of both sample and standard and then dividing the sample result by the standard result. The result was then multiplied by the standard concentration and a dilution factor to give sample creatinine in gdl^{-1}. The sample creatinine was then converted to mM for expressing with urinary PPi results.

3.10 Metabolic screening.

Blood screening tests in metabolic patients including: calcium, alkaline phosphatase, ferritin, magnesium and thyroid function were all carried out in the clinical biochemistry laboratories of the City Hospital.

3.11 Articular cartilage organ culture.

3.11.1 Sample collection.

Samples of articular cartilage were obtained only from subjects in the age-range 50-80 years; that appropriate for CPPD crystal deposition. The aim was to collect knee cartilage from two sources: joint tissues removed during knee surgery (pyrophosphate arthropathy, osteoarthritic and rheumatoid cartilage) and knee cartilage removed from human cadavers within 24 hours of death (pyrophosphate arthropathy, osteoarthritic and normal cartilage).

Tissue was transported from the operating theatre as soon as possible after removal from the subject. The joint tissues were transported in phosphate buffered saline (PBS) (Sigma) to the laboratory. When a knee joint was obtained from a cadaver, the whole joint was transported to the laboratory within 24 hours of death. Slab radiographs were taken of cadaveric knees to assess any joint damage.

3.11.2 Organ culture.

Articular cartilage was sliced from the femoral and tibial condyles of the knee joints using a sterile scalpel (BDH) in 9cm petri dishes (Gibco). Fibrocartilage was also used when present. Cartilage fragments were then washed several times in

PBS to remove any blood or soft tissue. The cartilage was then chopped into small fragments (2-3mm across). All organ culture work was carried out in a class II flow hood using aseptic technique.

Approximately 100mg of cartilage fragments were incubated in 1ml of incubation mixture in 24-well tissue culture plates (Gibco). The incubation mixture contained: minimum essential medium with Earle's salts (Gibco) supplemented with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). To this incubation mix tracer ^{32}P-PPi plus 100μM ATP (Sigma) was added for the determination of PPi hydrolysis and for the measurement of chondrocyte NTPP activity respectively. Samples were incubated at 37°C in a humidified incubator gassed with 5% CO_2/95% air.

The tissue samples were incubated for periods of 2 hours, 4 hours and 6 hours with a 0 hour control for each. Samples were in duplicate for each time course. Controls consisted of (a) tissue incubated in the absence of ATP and (b) incubation medium in the absence of cartilage. At the end of each time course, incubation medium and cartilage fragments were separated by centrifugation (200g for 10 minutes). Incubation medium was then frozen at -20°C for PPi and NTPP assays and cartilage fragments were maintained in incubation mixture for cell viability studies and the assay of DNA.

3.11.3 Cell count and viability.

Chondrocytes were released from the cartilage matrix by the following digestions:

1. Hyaluronidase (1mg/ml) (Sigma) in PBS for 15 minutes at room temperature.

2. Trypsin (0.25% (w/v)) (Gibco) for 30 minutes at 37°C.

3. Bacterial collagenase (Calbiochem) (3mg/ml in MEM + 10% FCS + Penicillin (100 U/ml) + Streptomycin (100 μg/ml)) overnight at 37°C.

The tissue was washed 3 times with PBS between each incubation. Chondrocytes were then washed free of collagenase by centrifugation (200g, 10 mins, 1200 rpm) and re-suspended in PBS twice. The final pellet was resuspended in MEM + 10% FCS + pen/strep. Cell numbers were quantified using an improved Nebaur haemocytometer, whilst cell viability was tested using the trypan blue exclusion method (Phillips 1973). The cellularity of cartilage samples was calculated by dividing the number of cells released by the wet weight of the cartilage. Remaining samples were centrifuged at 200g for 10 minutes and the resulting pellet resuspended in 1ml distilled water and frozen at -20°C until required for the assay of DNA.

3.11.4 Assay of chondrocyte DNA.

The fluorometric method used to estimate DNA in cartilage samples in this study was developed by Kissane and Robins (1958). The following reagents were used in the assay of chondrocyte DNA: a) 3,5-diaminobenzoic acid (DABA) (Sigma) 450mg/ml distilled water decolourized with 150mg/ml charcoal (BDH) overnight in the dark, then centrifuged and filtered through 0.22 μm durapore filter; b) DNA standards (calf thymus) (Sigma). A stock concentration of 200μg/ml was made for each assay. 100μl duplicates of each sample (diluted 1 in 10) and standards (0-100μg/ml) are evaporated to dryness at 60°C. 100μl of the decolourized DABA is added to each tube, and incubated for 45 mins at 60°C to hydrolyse the DNA. 1.5ml of 1.0M HCL (BDH) was then added to each tube and the fluorescence measured immediately at an excitation wavelength of 401nm and an excitation wavelength of 518nm. Sample DNA concentrations were then calculated from the resulting standard curve.

3.11.5 Assay of inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity in cartilage organ culture media.

The same methods were employed as for synovial fluid, the standard curve being adjusted accordingly.

3.12 Statistical methods.

Independent groups were compared by the Mann-Whitney U test. Test for association was by Spearman's rank correlation.

3.13 Expression of results.

Independent groups of data were expressed as the mean (±sem), where the data was found to be normally distributed. Where data was not normally distributed results were expressed as the median (± interquartile range).

CHAPTER 4: Quantification of synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity: methodological variables.

4.1 Introduction.

As this study employs a method previously applied to the quantification of intracellular PPi and PPi in cell culture media (McGuire *et al* 1980(b)), several methodological variables were studied to ensure the accuracy and specificity of this assay when applied to synovial fluid.

4.2 The linearity and reproducibility of the inorganic pyrophosphate standard curve.

The PPi standard curve increased in a linear fashion from 0.5μM through 4μM as shown in figure 6. The lack of linearity above 4μM could be due to insufficient incubation time for PPi to be completely converted to 6-phosphogluconate. Since synovial fluid PPi always measured well below 6μM on the curve, the need for a longer incubation time was unnecessary. Figure 7a shows three standard curves in the concentration range 0.5-8μM exhibiting the linearity up to 4μM and the reproducibility of the PPi standard curve measured on the same day. Whereas figure 7b illustrates the reproducibility of the PPi standard curve on three separate days.

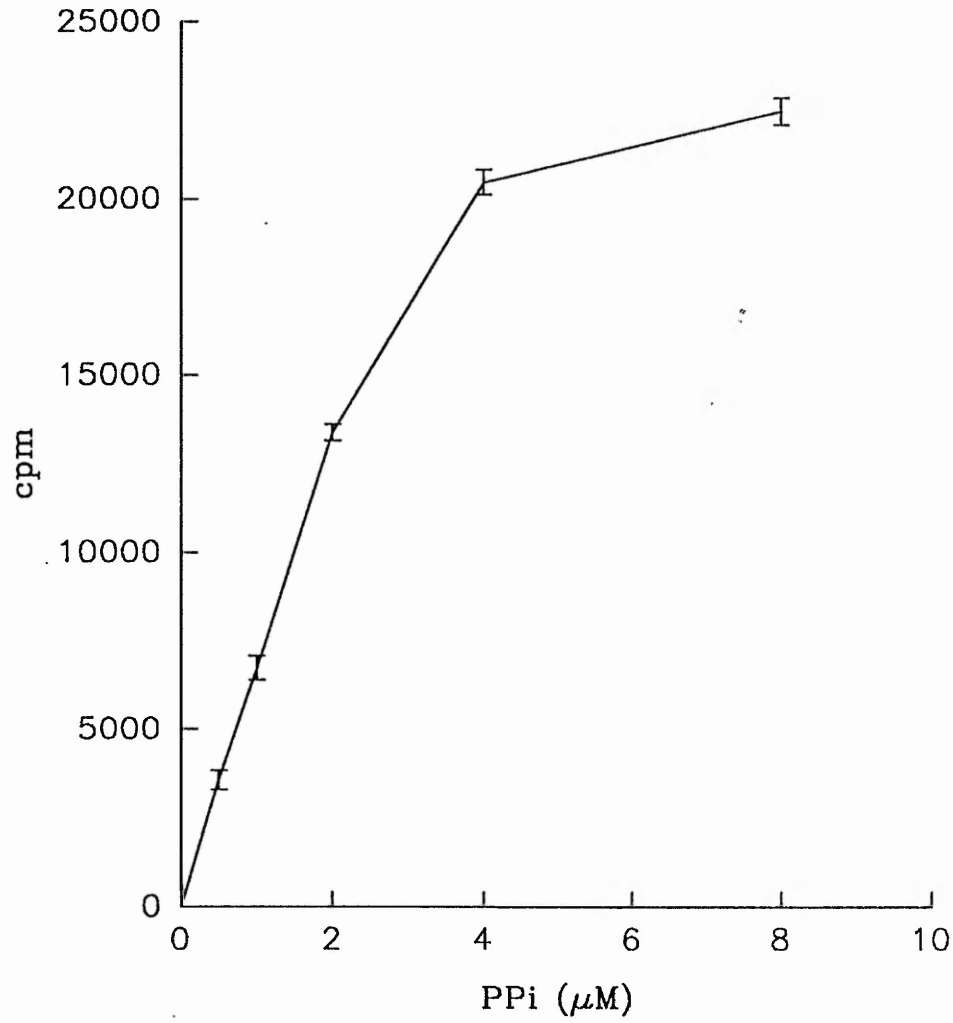


Figure 6: PPi standard curve. Results represent the mean (±sem) of triplicates.

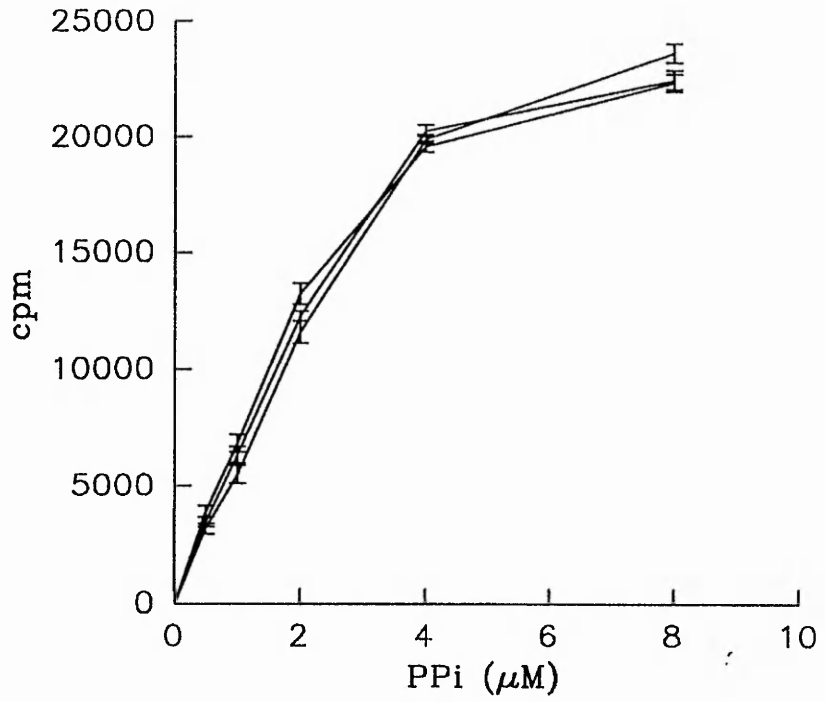


Figure 7a: 3 PPi standard curves measured on the same day. Results represent the mean (±sem) of triplicates.

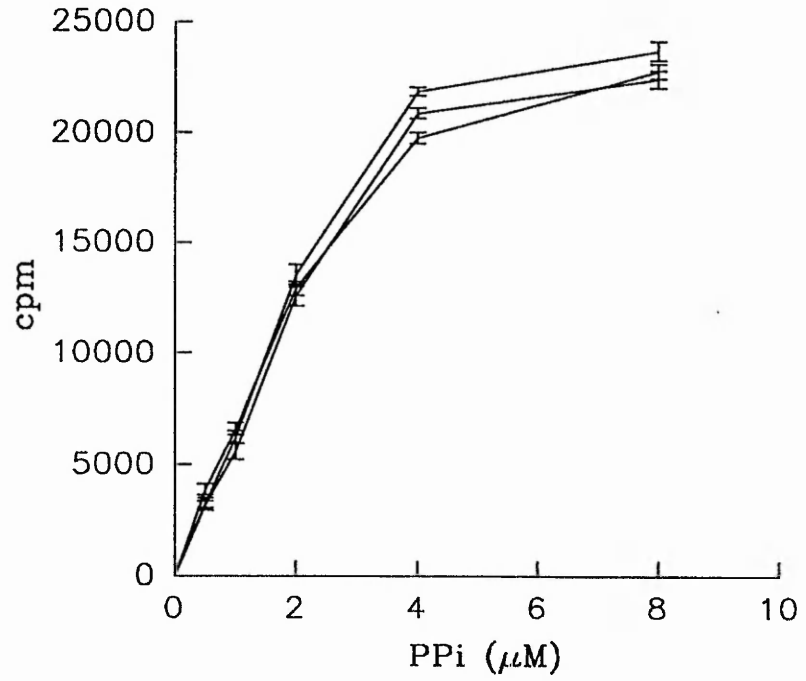


Figure 7b: 3 PPi standard curves measured on three separate days. Results represent the mean (±sem) of triplicates.

4.3 Recovery of inorganic pyrophosphate added to synovial fluid.

When varying quantities of standard PPi were added to the same synovial fluid sample divided into 5 aliquots, recovery was quantitative as shown in table 3. Confirming the accuracy of this assay. Results are the mean of duplicate estimations.

4.4 The reproducibility of the inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase assays.

To test the reproducibility of the PPi and NTPP assays, a single synovial fluid sample was divided into 10 aliquots in which both PPi (μM) and NTPP activity (μmol PPi/min/mg protein) were measured. The resulting coefficient of variation was 13% (mean = 14.7 μM, sd = 1.64) for PPi and 12.9% (mean = 0.080 μmol PPi/min/mg protein, sd=0.008) for NTPP activity.

4.5 ATP concentration optimum for maximum inorganic pyrophosphate generation.

The production of PPi increased up to an ATP concentration of 100μM, above which PPi concentrations started to decrease as shown in figure 8. This finding

Baseline PPi (μM)	PPi added (μM)	Measured PPi (μM)	% Recovery
6.4	8.0	15.4	107
6.4	4.0	10.5	101
6.4	2.0	8.2	97
6.4	1.0	8.2	111
6.4	0.5	7.3	106

Table 3: Recovery of PPi added to synovial fluid

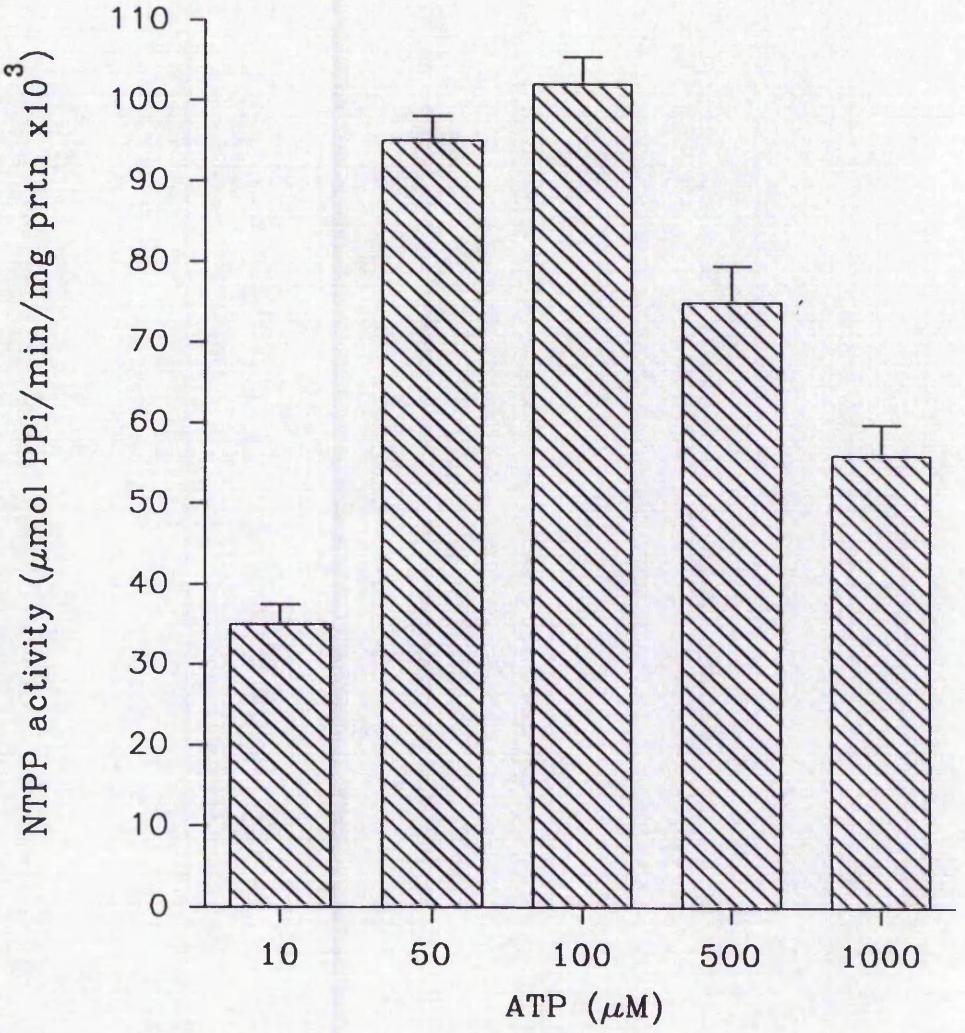


Figure 8: PA synovial fluid NTPP activity under varying concentrations of ATP. Results represent the mean (±sem) of triplicates.

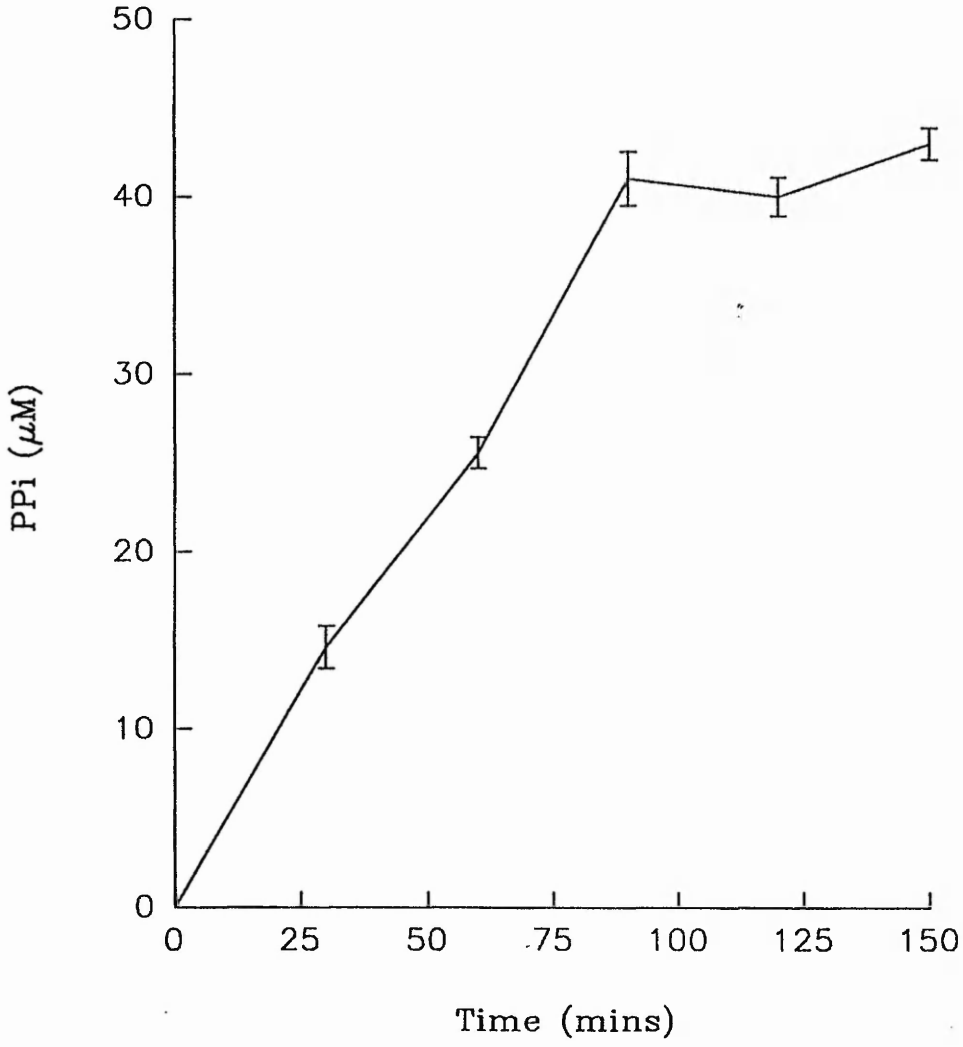


Figure 9: Production of PPi in PA synovial fluid over time, in the presence of 100μM ATP. Results represent the mean (±sem) of triplicates.

was regardless of disease category. The generation of PPi in the presence of 120μM ATP was linear up to 90 minutes after which it started to plateau as shown in figure 9.

4.6 Discussion.

The method employed in this study for the estimation of synovial fluid PPi is a modification of a method previously developed for the measurement of PPi in cell cultures (McGuire *et al* 1980(b)). This radiometric method, sensitive to a concentration of 10 picomoles, utilizes an enzyme cascade reaction incorporating UDP-[^{3}H]-glucose and converting it into the reaction product 6-phospho-[^{3}H]-gluconic acid, illustrated in figure 5, chapter 3.3. In this study the estimation of PPi was applied to knee synovial fluid in order to measure free PPi concentrations and to measure the activity of the ecto-enzyme NTPP which catalyses the conversion of NTP to NMP and PPi.

As described in detail in chapter 3.3, this method includes controls for any hydrolysis of PPi during the incubation and extraction procedures. It was evident, however, that recovery of synovial fluid PPi after incubation and extraction procedures was typically greater than 90%. To measure any reaction products from substrates other than PPi, enzyme blank controls were included by omitting UDPG-pyrophosphorylase. Although such reaction products were minimal, the true

sample PPi value can be calculated by subtracting the enzyme blank result.

The estimation of NTPP activity in synovial fluid is by the generation of PPi in the presence of ATP as described in chapter 3.5. These data show that an optimum concentration of 100μM ATP was required for maximum activity of synovial fluid NTPP; above this a slight decrease in enzyme activity was apparent. Initial experiments were carried out using an ATP concentration of 120μM, since this ATP concentration did not produce results that differed from using 100μM, 120μM ATP was used in subsequent experiments. Since PPi production was linear up to 90 minutes, an incubation time of 30 minutes was carried out, to reduce overall assay time, in the presence of 120μM ATP for each NTPP assay.

The linearity and reproducibility of the PPi standard curve and the quantitative recovery of PPi when added to synovial fluid demonstrate the accuracy and specificity of this assay when applied to synovial fluid in our laboratory. In addition, the low coefficients of variation for both PPi levels and NTPP activity exhibit the accurate reproducibility of these assays.

CHAPTER 5: Synovial fluid inorganic pyrophosphate concentrations and nucleoside triphosphate pyrophosphatase activity of diseased and normal knees.

5.1 Synovial fluid inorganic pyrophosphate concentrations and nucleoside triphosphate pyrophosphatase activity of diseased and normal knees.

In order to assess any disturbance of articular PPi metabolism in arthritic disease groups, this study measured knee synovial fluid PPi in pyrophosphate arthropathy, osteoarthritis, rheumatoid arthritis and in normal controls. The activity of synovial fluid NTPP was also measured in the above groups to assess the role of this enzyme in the production of articular PPi and how NTPP activity compares between disease groups and normals.

Figures 10 and 11 show synovial fluid PPi and NTPP results for disease groups and normals. One synovial fluid sample was collected from each subject.

The rank order of both mean synovial fluid PPi concentrations and mean NTPP activity for disease groups was: pyrophosphate arthropathy > osteoarthritis > rheumatoid arthritis, with significant differences between all groups. PPi levels were significantly higher in pyrophosphate arthropathy compared to normal ($p<0.001$), whilst no significant differences were observed between subjects with osteoarthritis and normal. In rheumatoid arthritis, synovial fluid PPi levels were significantly lower than normal ($p<0.001$). However, no significant difference was

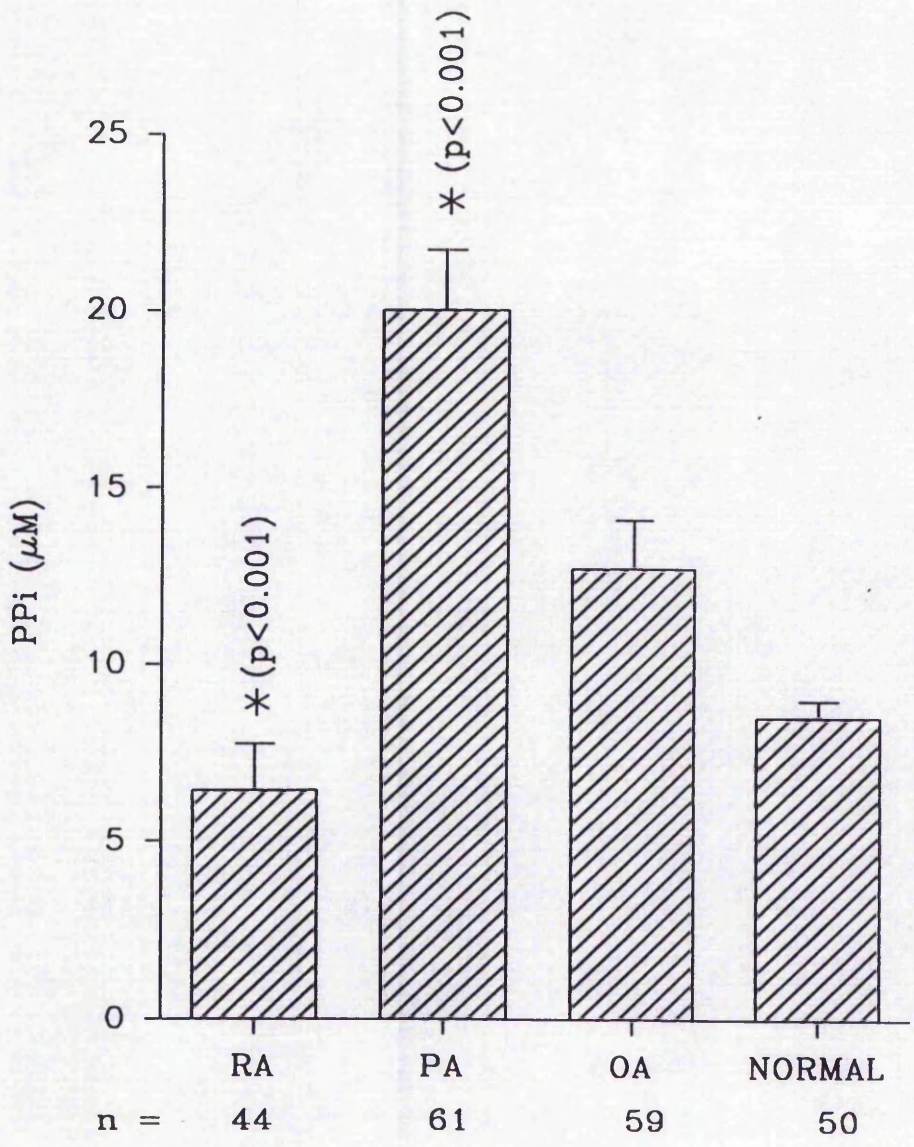


Figure 10: Synovial fluid PPi levels of disease groups and normals. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to the normal control group, whilst p indicates the level of significance.

RA = rheumatoid arthritis; PA= pyrophosphate arthropathy; OA= osteoarthritis.

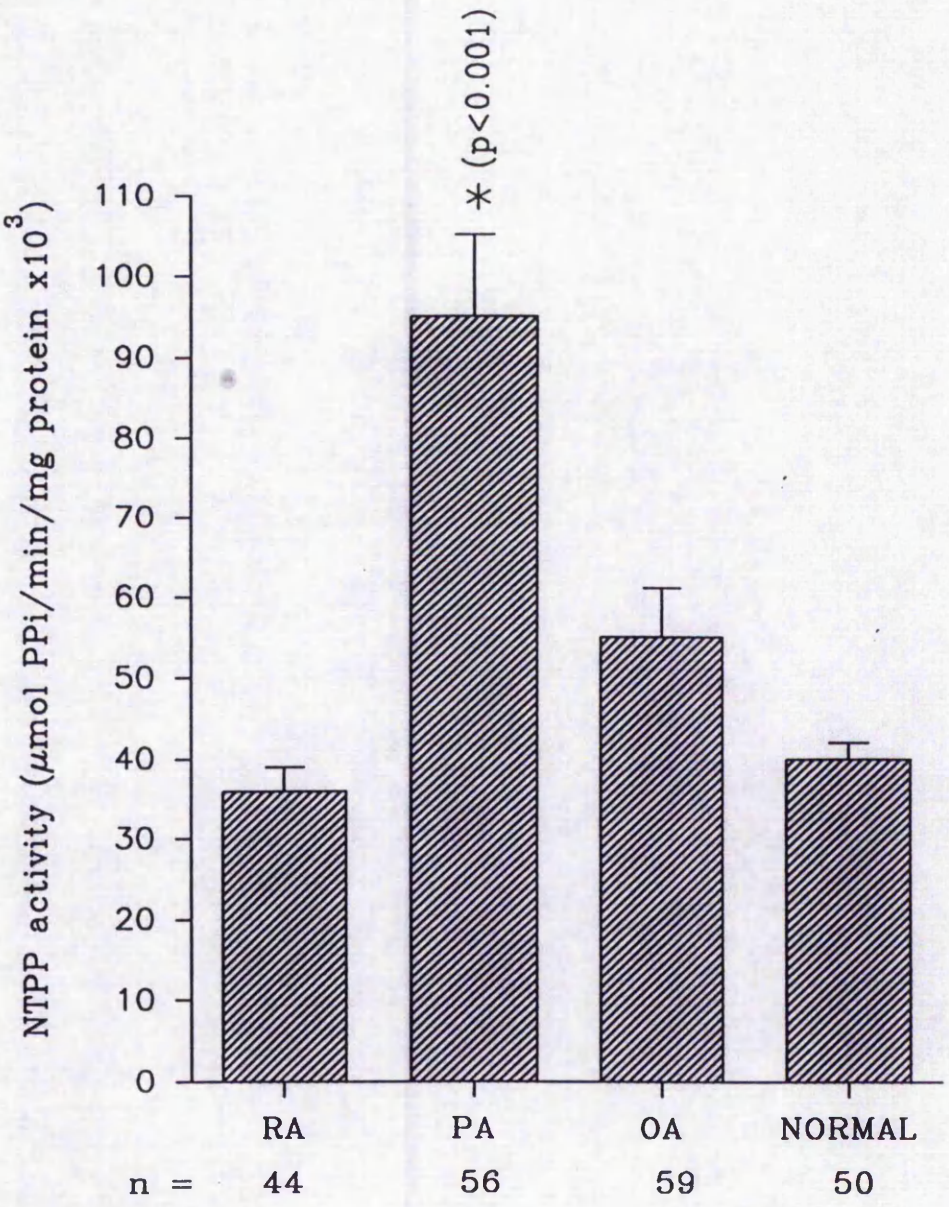


Figure 11: Synovial fluid NTPP activity of disease groups and normals. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to the normal control group, whilst p indicates the level of significance.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis.

observed for NTPP activity between rheumatoid arthritis and normal.

A significant positive correlation was observed between synovial fluid PPi and NTPP activity for all subjects together: $n = 128$, $r = 0.3$, $p < 0.01$. As illustrated in figure 12.

5.2 Synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity of inflamed and non-inflamed knees.

The degree of clinical inflammation was assessed in abnormal knees, as described in chapter 2.2. To study the effect of clinical inflammation on synovial fluid PPi levels and NTPP activity, and how this may effect the outcome of synovial fluid studies. "Active" and "inactive" knees were compared within diagnostic categories.

Figures 13 and 14 show the comparisons between "active" and "inactive"groups within disease categories for synovial fluid PPi levels and NTPP activity. Differences were observed in pyrophosphate arthropathy, where synovial fluid PPi was significantly lower in the "active" compared to the "inactive" group, ($p<0.001$). In rheumatoid arthritis PPi was higher in the "active" compared to the "inactive" group, ($p<0.001$); conversely NTPP activity was lower in the "active" compared to "inactive" group in rheumatoid arthritis ($p<0.01$). In osteoarthritis, no significant differences for PPi concentrations or NTPP activity were observed

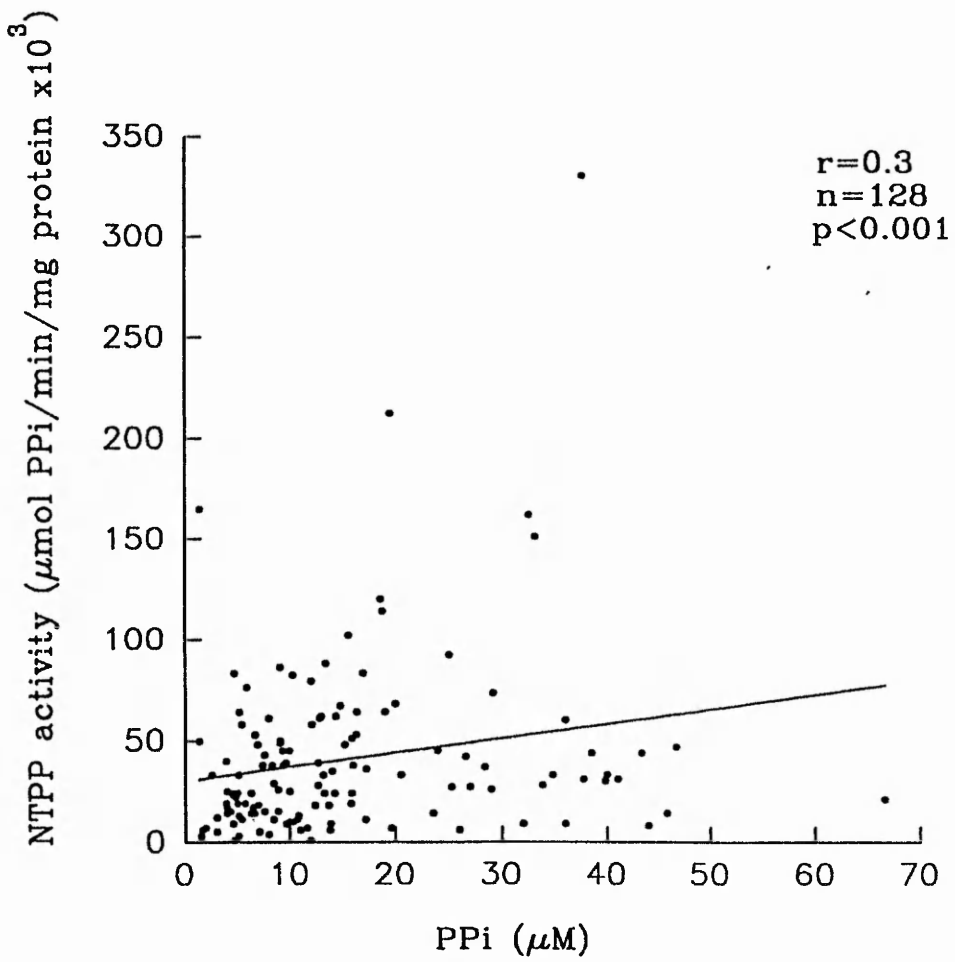


Figure 12: Correlation between synovial fluid PPi levels and NTPP activity for all disease groups together. Where n denotes sample number, r the coefficient of correlation and p the level of statistical significance.

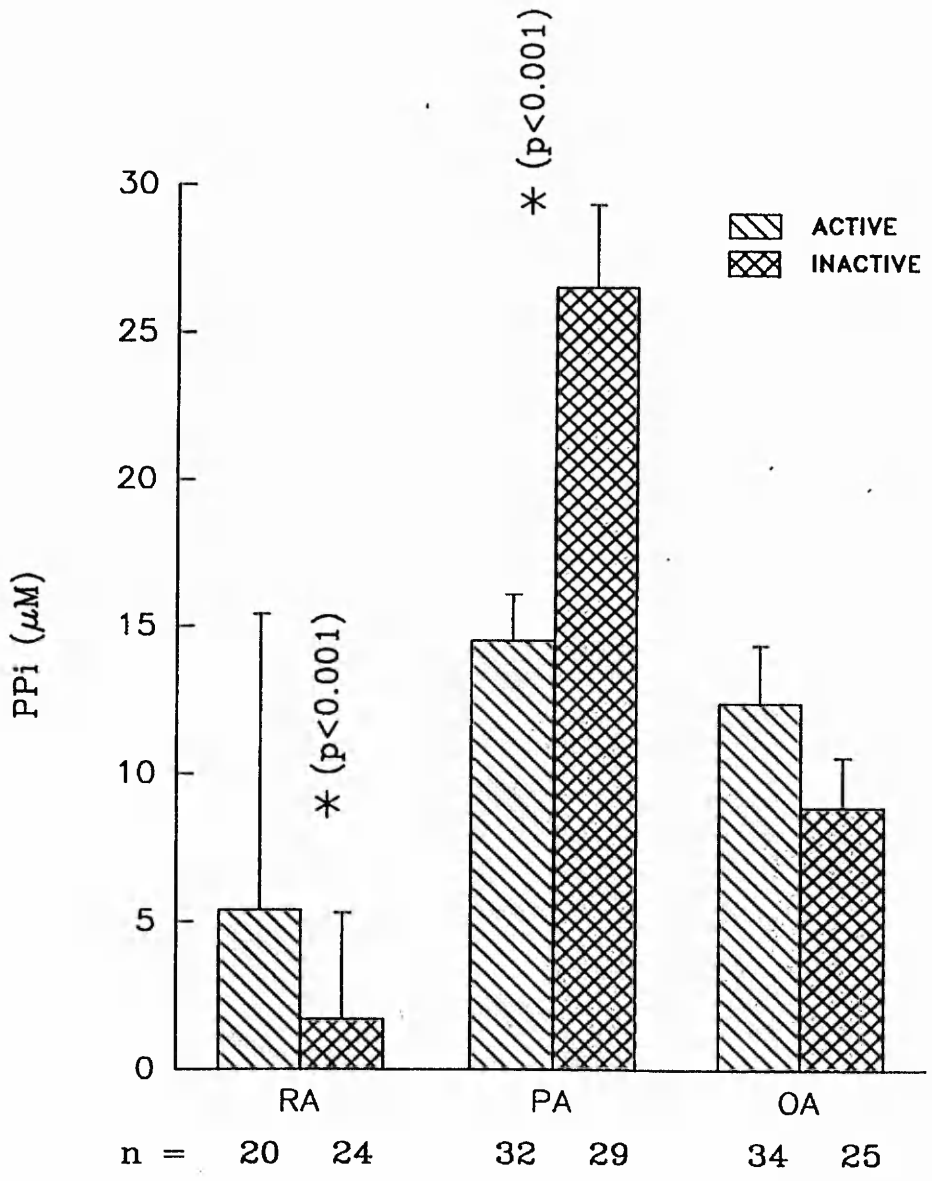


Figure 13: Synovial fluid PPi levels in "active" and "inactive" knees of disease groups. Results represent the mean (±sem) for all groups except RA, where the results are expressed as the median (±interquartile range). n denotes subject number in each group. * indicates statistical significance between "active" and "inactive" knees, whilst p indicates the level of significance.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis.

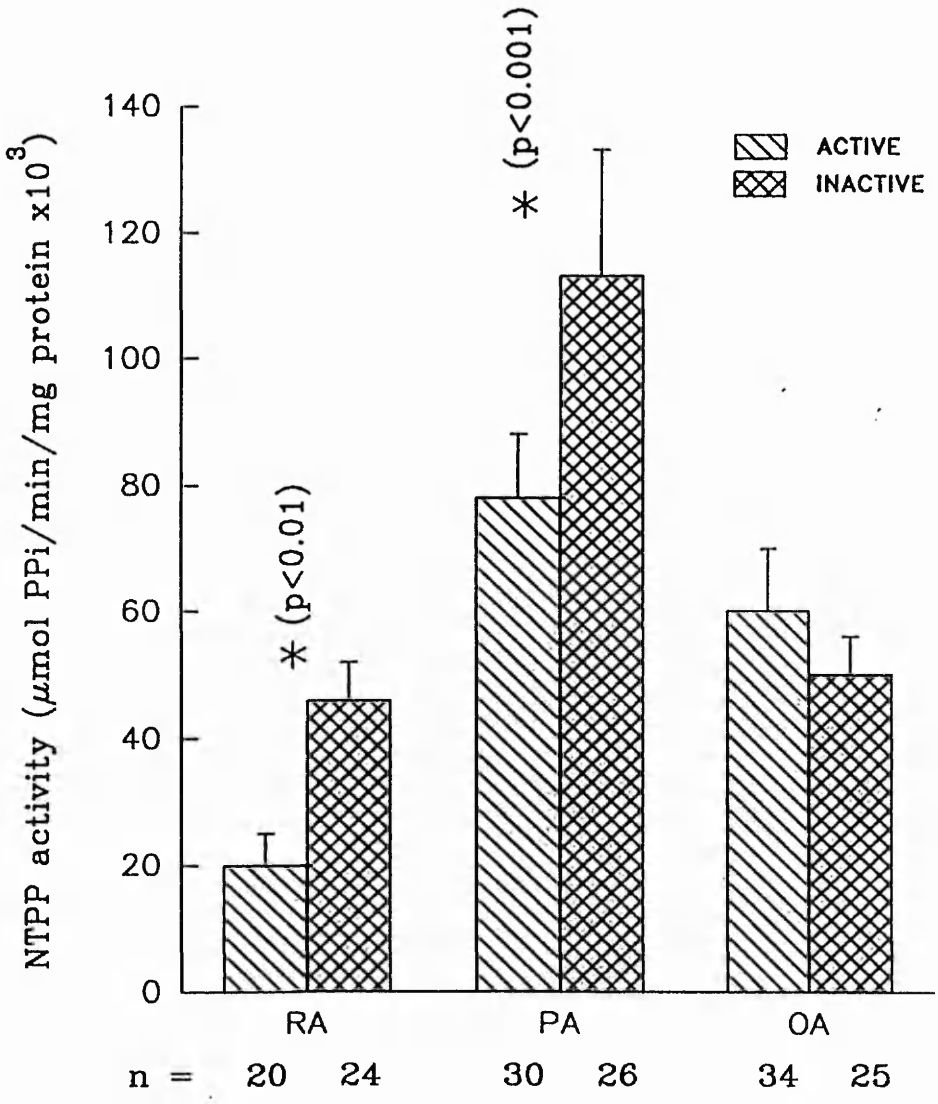


Figure 14: Synovial fluid NTPP activity in "active" and "inactive" knees of disease groups. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance between "active" and "inactive" knees, whilst p indicates the level of significance.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis. between the "active" and "inactive" groups.

5.3 Synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity of normal knees.

Although CPPD crystal deposition exhibits a strong association with age, it is not known whether an abnormality of PPi metabolism exists in the non-diseased aged joint, favouring CPPD crystal deposition. In this study the activity of synovial fluid NTPP and PPi concentrations were measured in normal knees from subjects covering a broad age range to assess any effects of ageing and whether these may associate with increased CPPD crystal deposition.

In the normal group, 4 subjects over age 50 were found to have isolated radiographic chondrocalcinosis (i.e no associated structural changes of arthropathy). These 4 subjects together with 1 subject with no chondrocalcinosis had synovial fluid CPPD crystals. This group of 5 subjects were termed asymptomatic and excluded from the normal group and analysed separately.

Normal synovial fluid PPi levels and NTPP activity are compared to the disease groups in figures 10 and 11 and any differences are described above in section 5.1. Neither PPi levels or NTPP activity showed any significant correlation with age in the normal group: $r = -0.04$ and $r = 0.12$ respectively. In the small group of asymptomatic synovial fluids which were found to contain CPPD crystals, both PPi and NTPP activity were significantly increased compared to non-crystal containing normal fluids ($p<0.001$) as shown in figures 15 and 16.

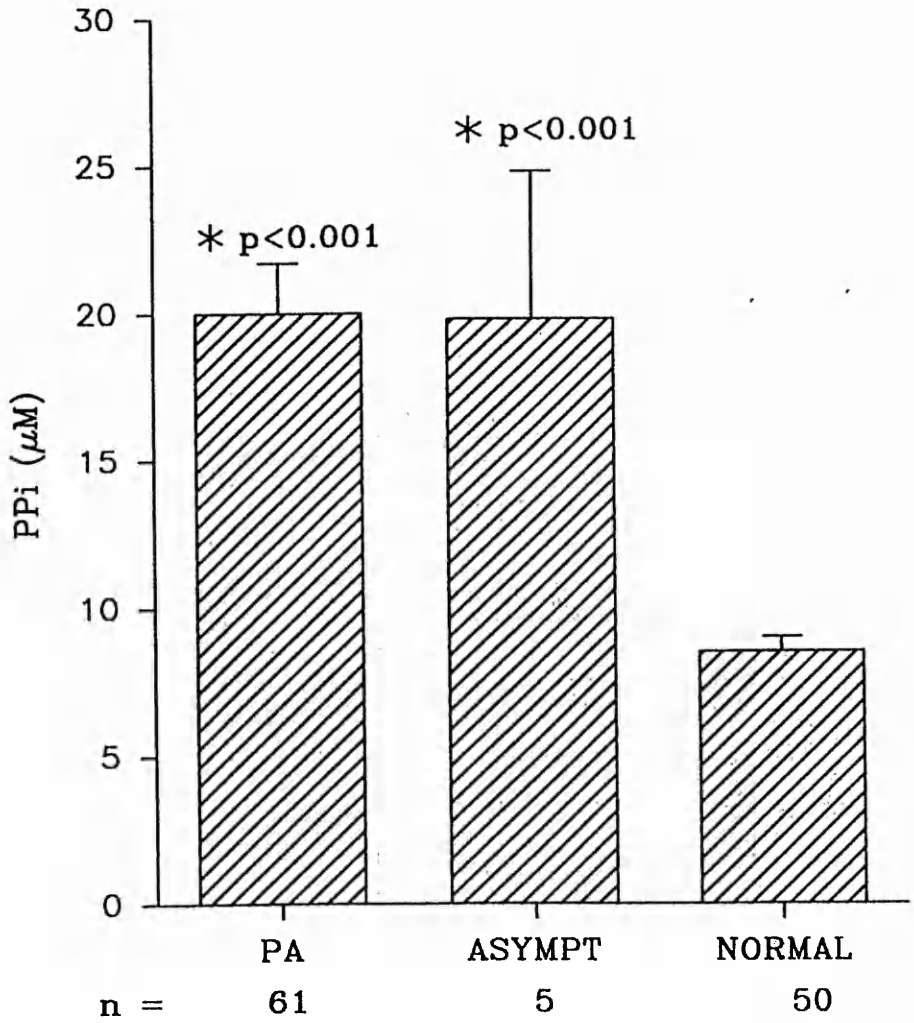


Figure 15: Synovial fluid PPi levels of asymptomatic, normal and pyrophosphate arthropathy knees. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to normal, whilst p indicates the level of significance.

Asympt = asymptomatic. PA = pyrophosphate arthropathy.

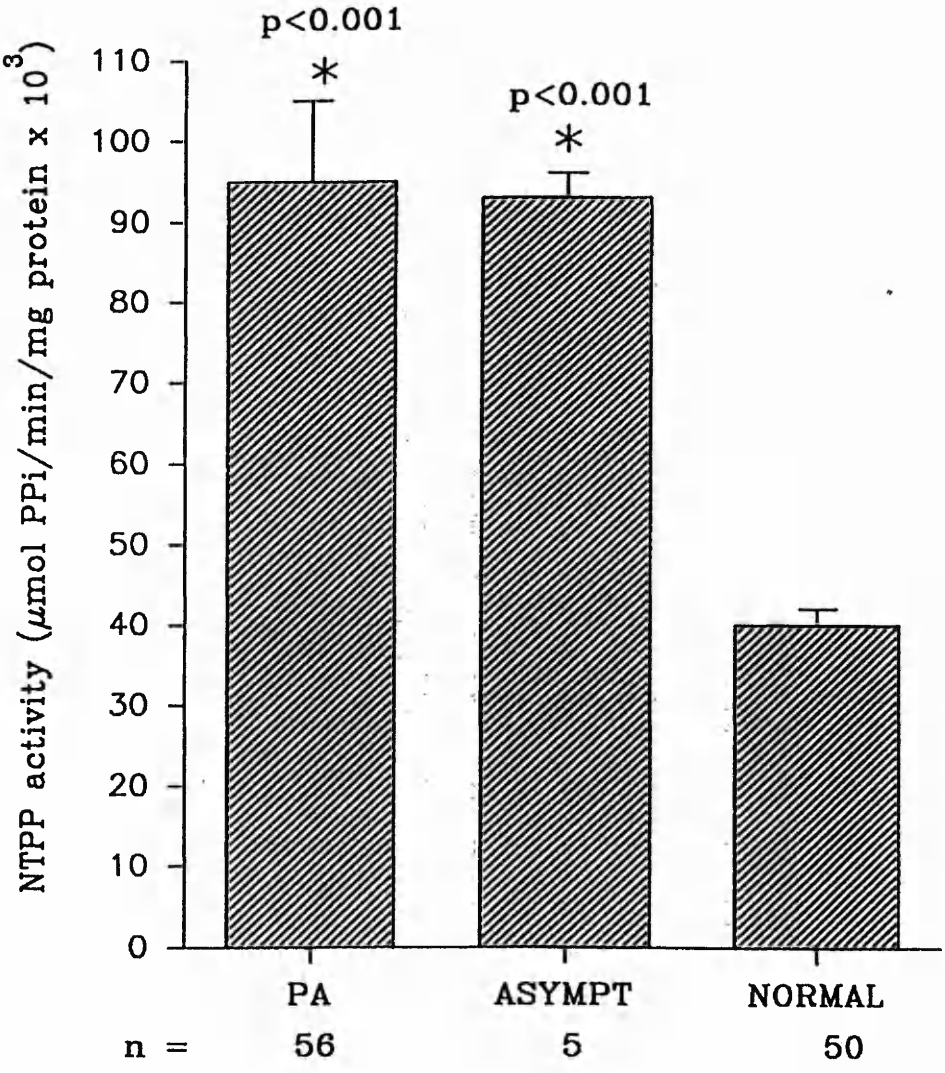


Figure 16: Synovial fluid NTPP activity of asymptomatic, normal and pyrophosphate arthropathy knees. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to normal, whilst p indicates the level of significance.

Asympt = asymptomatic. PA = pyrophosphate arthropathy.

5.4 DISCUSSION.

Increased concentrations of synovial fluid PPi have previously been reported in association with CPPD crystal deposition and in the pathogenesis of pyrophosphate arthropathy (Russell 1970, McCarty *et al* 1971, Altman *et al* 1973(a), Silcox *et al* 1974, Pattrick *et al* 1991). As normal urinary (Russell *et al* 1970, Pflug *et al* 1969, Camerlain *et al* 1980) and plasma (Russell *et al* 1970, Ryan *et al* 1979) PPi levels are evident in pyrophosphate arthropathy, localised production of PPi within the joint has been proposed. Articular cartilage has been implicated as the source of synovial fluid PPi as it has been shown to elaborate PPi in culture, whilst other articular tissues have not (Howell *et al* 1975, Ryan *et al* 1981). As cartilage extracellular fluid and synovial fluid are thought to be in equilibrium (Rachow and Ryan 1988), synovial fluid, therefore, provides an ideal medium in which to measure changes in PPi metabolism existing within the cartilage matrix.

The results of significantly elevated synovial fluid NTPP activity and PPi concentrations in pyrophosphate arthropathy compared to osteoarthritis and rheumatoid arthritis confirm previous findings (Russell *et al* 1970, Silcox and McCarty 1974, Rachow and Ryan 1985(a)), and parallel results from cartilage experiments (Howell *et al* 1975. Tenenbaum *et al* 1981). In addition the positive correlation found between NTPP activity and PPi levels in synovial fluid for all disease groups together, supports the hypothesis that this enzyme is a major contributor to the localised increase in synovial fluid PPi levels found within

affected joints.

Since articular cartilage in culture releases NTPP into culture media (Rachow *et al* 1985) this would seem a likely source for synovial fluid NTPP. The reason for increased cartilage and synovial fluid NTPP activity in sporadic pyrophosphate arthropathy is uncertain although several ideas have been postulated, as introduced in chapter 1.5.

It is thought unlikely that CPPD crystal dissolution contributes to increased synovial fluid PPi levels in pyrophosphate arthropathy, due to the high turnover rate of synovial fluid PPi compared to the slow dissolution rate of crystals (Camerlain *et al* 1975). Undetermined synovial fluid volume in the joint is also a potential confounding factor in the estimation of PPi concentrations. However, any relationship between synovial fluid PPi and joint volume has not been established (Altman 1973(a)).

In osteoarthritic synovial fluid NTPP activity and PPi levels, although elevated, did not differ significantly from the normal group in this study. Osteoarthritic cartilage in organ culture has been shown to elaborate more PPi than normal cartilage (Howell 1975). This study is the first to include an extensive group of normal controls, perhaps therefore, reflecting a more realistic normal synovial fluid mean PPi level. Normal PPi and NTPP activity in osteoarthritis would support the hypothesis that increased synovial fluid NTPP activity and PPi levels found in

joints affected by pyrophosphate arthropathy are responsible for the promotion of CPPD crystal deposition which is not present in uncomplicated osteoarthritis.

Decreased levels of PPi and NTPP activity found in rheumatoid synovial fluid compared to that in pyrophosphate arthropathy, and the significantly lower levels of PPi found in rheumatoid arthritis compared to normal supports the negative association found between CPPD crystal deposition and rheumatoid arthritis (Doherty *et al* 1984). Factors associated with the inflammatory process may contribute to this result, as previously described in chapter 1.3 and 1.5.

Reduced synovial PPi concentrations exist in the presence of normal NTPP activity in the rheumatoid group, suggesting that the enzyme is perhaps less affected than the ion by the inflammatory processes of rheumatoid arthritis, for example increased pyrophosphatase activity in the rheumatoid joint may promote decreased PPi levels. The normal NTPP activity cannot be attributed to the inflammatory infiltrate as the white blood cell count of synovial fluid has been shown to correlate negatively with NTPP activity in synovial fluid (Rachow and Ryan 1985(a)). It could be that NTPP activity is inhibited in the inflamed joint, reflected by the reduced synovial fluid PPi levels. Activity may then recover in the assay system, possibly by the *in vitro* dilution of an inhibitor, producing the more normal levels of NTPP activity found.

The findings of reduced synovial fluid NTPP activity and PPi levels in rheumatoid

arthritis and acute pseudogout compared to those found in chronic pyrophosphate arthropathy (Silcox and McCarty 1974) suggest an association between the inflammatory state of the joint and synovial fluid PPi concentrations. Since the clinical inflammatory state of individual joints varies widely within disease groups, it is of interest how this affects synovial fluid PPi levels in osteoarthritis and pyrophosphate arthropathy as well as the more inflammatory rheumatoid arthritis and pseudogout.

Although there is not an established method of assessing the clinical inflammatory state of individual joints, the system used in this study, described in chapter 2.2, has been shown to correlate with complement activation and synovial fluid white blood cell count in several arthritides (Doherty *et al* 1988, Hamilton *et al* 1990).

The effects of clinical inflammation on synovial fluid NTPP activity and PPi levels within affected joints were found to vary between disease groups. In pyrophosphate arthropathy synovial fluid PPi levels were significantly reduced in the "active" compared to "inactive" joints. Although a similar reduction of NTPP activity was apparent in the "active" compared to the "inactive" group in pyrophosphate arthropathy, the difference was not statistically significant. The reduced synovial fluid PPi in "active" joints may be a reflection of inflammatory effects. For example increased clearance of PPi from the joint via increased blood flow and increased activity of synovial fluid pyrophosphatases. No differences were detected between "active" and "inactive" joints in osteoarthritis, suggesting

the existence of different inflammatory mechanisms in osteoarthritis compared to pyrophosphate arthropathy. One possible explanation may be the presence of CPPD crystals in pyrophosphate arthropathy and their inflammatory effects.

The contrasting results in the rheumatoid group of increased synovial fluid PPi in the "active" compared to the "inactive" group and increased NTPP activity in the inactive compared to the active group, suggest that as with osteoarthritis, differing effects on PPi metabolism exist within the rheumatoid joint compared to the pyrophosphate arthropathy joint.

The significant effects of clinical inflammation on synovial fluid PPi levels and NTPP activity in disease groups, allowing a more accurate interpretation of the results, support the inclusion of such an assessment of clinical inflammation in future synovial fluid studies.

Pyrophosphate arthropathy and osteoarthritis are both strongly associated with age (Felson *et al* 1987, McCarty 1976, Doherty *et al* 1988). However, in the large group of normal subjects studied, no significant correlation was found between synovial fluid PPi levels or NTPP activity with age. It is implied, therefore, that ageing alone is not responsible for the increased synovial fluid NTPP activity or PPi levels found in pyrophosphate arthropathy. Factors in addition to age therefore must be involved in the promotion of increased NTPP activity and articular PPi concentrations, and hence predisposition to CPPD crystal deposition.

The finding of increased synovial fluid PPi and NTPP activity in the small group of asymptomatic "normal" joints containing CPPD crystals again associates increased NTPP activity and PPi levels in the synovial fluid with CPPD crystal deposition. In this instance, however, in the absence of co-existing disease. It is evident, therefore, that in addition to the presence of chondrocalcinosis, "shedding" of crystals into the synovial fluid can also occur in the absence of any symptoms. CPPD crystals may, therefore, exist as "innocent bystanders", perhaps only having a pathogenic effect in the presence of co-existing joint disease.

CHAPTER 6: Synovial fluid alkaline phosphatase activity.

6.1 Synovial fluid alkaline phosphatase activity of diseased and normals knees.

As alkaline phosphatase is a major pyrophosphatase, a deficiency of this enzyme in articular cartilage could lead to increased PPi and the promotion of CPPD crystal deposition. In this study, knee synovial fluid ALP was measured in subjects with pyrophosphate arthropathy, osteoarthritis and rheumatoid arthritis. In addition ALP was also measured in a large group of normal synovial fluids.

ALP activity was compared between disease groups and the normal controls in order to determine whether this synovial fluid enzyme demonstrates an association with CPPD crystal deposition as previously proposed or with the inflammatory state of the joint as suggested in rheumatoid arthritis.

Synovial fluid ALP activity was found to be highest in rheumatoid arthritis where activity was significantly higher than that found in pyrophosphate arthropathy ($p<0.001$), osteoarthritis $p<0.001$) and normal ($p<0.001$) as shown in figure 17. Synovial fluid ALP activity was increased in pyrophosphate arthropathy compared to osteoarthritis ($p<0.001$) and normal ($p<0.001$). Synovial fluid ALP activity in osteoarthritis was not significantly different to normal.

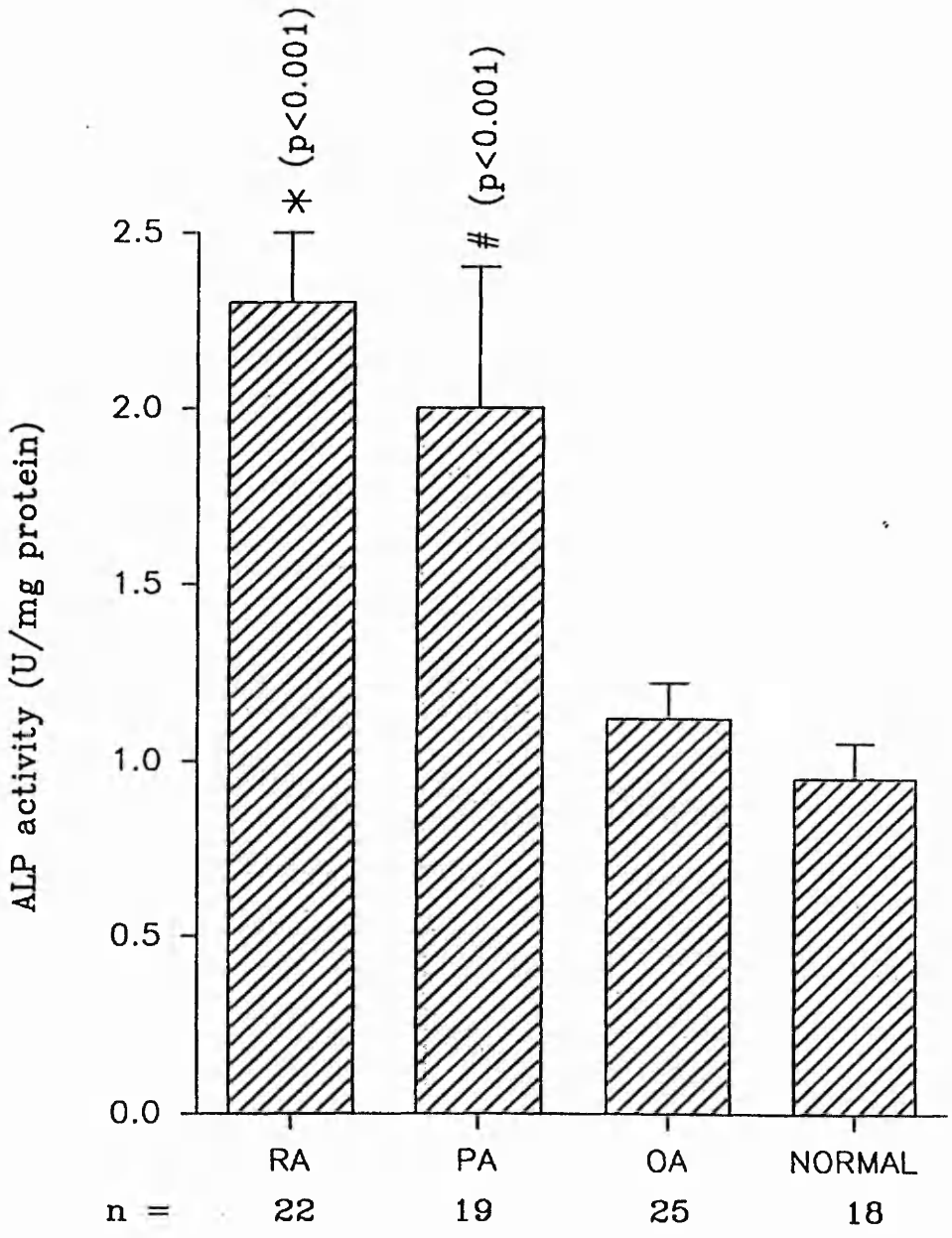


Figure 17: Synovial fluid ALP activity of disease groups and normals. Results represent the mean (±sem) for each group, n denotes subject number in each group. * ALP activity was significantly greater in RA compared to PA, OA and normal. # ALP activity was significantly greater in PA compared to OA and normal. p indicates the level of statistical significance.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis.

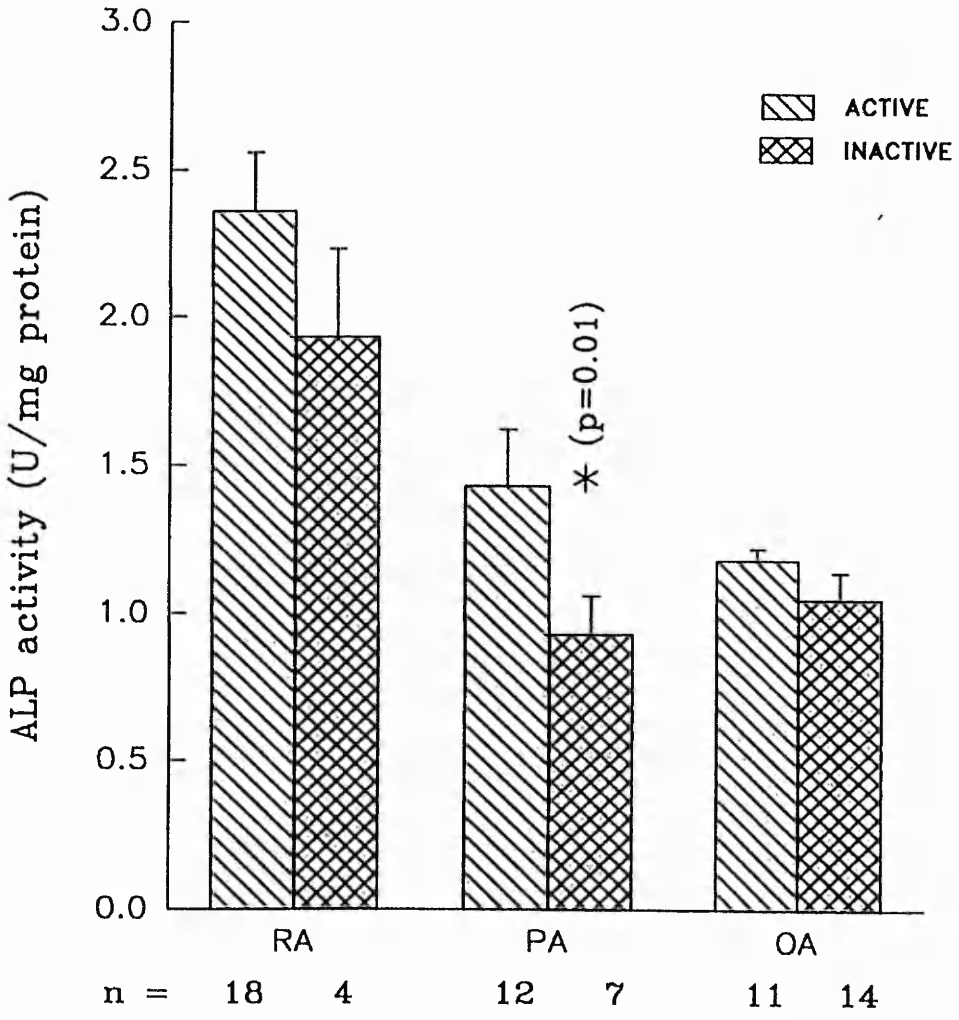


Figure 18: Synovial fluid ALP activity in "active" and "inactive" knees of disease groups. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance between "active" and "inactive" knees, whilst p indicates the level of significance found.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis.

6.2 Synovial fluid alkaline phosphatase activity of inflamed and non-inflamed joints.

To determine the effect of clinical inflammation on enzyme activity a comparison was made between "active" and "inactive" knees within disease categories. Knees were assessed as described in chapter 2.2.

In all disease groups synovial fluid ALP activity was always higher in the "active" compared to the "inactive" groups as shown in figure 18. However, statistical significance was observed only in pyrophosphate arthropathy ($p = 0.01$).

6.3 Discussion.

Previously, a deficiency in synovial fluid pyrophosphatase activity due to both glucose-6-phosphatase and ALP has been implicated in CPPD crystal deposition via effects on the hydrolysis of PPi (Russell *et al* 1970, Good and Starkweather 1969, Yaron *et al* 1970) as described in 1.7. Latterly, however, reports have disputed these earlier findings, suggesting no such deficiency of pyrophosphatase activity exists (McCarty *et al* 1971, Altman *et al* 1973(a), Giblisco 1985).

In this study as with more recent reports, a deficiency of synovial fluid ALP was not apparent in pyrophosphate arthropathy. Contrary to early reports, an increase

in the activity of this enzyme was found in pyrophosphate arthropathy when compared to osteoarthritis or normal controls. The earlier findings of decreased synovial fluid ALP activity in pyrophosphate arthropathy may have been due to the use of inflammatory synovial fluids from patients with rheumatoid arthritis as controls (Russell *et al* 1970, Yaron *et al* 1970), which as this and other studies show, contain increased ALP activity (Cimmino *et al* 1987). The source of ALP in rheumatoid synovial fluid is thought to be a local one from within the joint, possibly from synovial tissue where high ALP activity was found (Cimmino *et al* 1987).

When synovial fluids from clinically active joints were compared to inactive joints in this study, ALP activity was increased in every disease group, although the increase was only statistically significant in pyrophosphate arthropathy. This is suggestive of a possible association with synovial fluid ALP activity and the inflammatory state of the joint.

By the inclusion of normal synovial fluid controls, for the first time in this study, it was confirmed that PPi hydrolysis in pyrophosphate arthropathy synovial fluid is not at fault, at least in idiopathic chondrocalcinosis. Whether this finding is a reflection of ALP activity in the articular cartilage is not known. Several reports have suggested a deficiency of ALP activity within the cartilage matrix (Howell *et al* 1976, Tenenbaum *et al* 1981). It may be that localised ALP activity within the cartilage matrix is more pertinent to CPPD crystal deposition than synovial

fluid levels of enzyme activity, in respect of the influence of clinical inflammation on synovial fluid ALP activity.

The increased synovial fluid ALP activity found in rheumatoid joints and in the active compared to inactive groups imply an association with clinical disease activity. The elevated synovial fluid ALP activity in pyrophosphate arthropathy compared to osteoarthritis suggest a similar association with disease activity, possibly linked to the inflammatory effect of CPPD crystals on the synovium.

CHAPTER 7: Synovial fluid 5'nucleotidase activity.

7.1 Synovial fluid 5'nucleotidase activity of diseased and normals knees.

A putative role for increased articular 5'nucleotidase (5NT) activity in the promotion of CPPD crystal deposition in pyrophosphate arthropathy has previously been suggested (Tenenbaum *et al* 1981, Rachow *et al* 1988, Wortmann *et al* 1991) as introduced in chapter 1.6.

In an attempt to assess the relationship between synovial fluid 5NT activity and CPPD crystal deposition, 5NT activity was measured in knee synovial fluid from subjects with pyrophosphate arthropathy, osteoarthritis, rheumatoid arthritis and normals to determine whether an increase in 5NT activity exists in knees with pyrophosphate arthropathy. In addition, 5NT was measured in synovial fluid from asymptomatic knees (knees with synovial fluid CPPD crystals in the absence of any symptoms or clinical signs of disease), to determine effects on 5NT activity in the presence of CPPD crystal deposition, but in the absence of any obvious disease.

Synovial fluid 5NT activity was found to be significantly increased in pyrophosphate arthropathy compared to: rheumatoid arthritis ($p<0.05$); osteoarthritis ($p<0.001$) and normal ($p<0.001$). Results are shown in figure 19. 5NT activity was also significantly increased in rheumatoid arthritis compared to

that in osteoarthritis ($p<0.001$) and normals ($p=0.02$). 5NT activity was not significantly different in osteoarthritis compared to normal.

7.2 Synovial fluid 5'nucleotidase activity of asymptomatic knees.

Asymptomatic synovial fluid was obtained from the knees of subjects with or without radiographic chondrocalcinosis, but containing CPPD crystals in the absence of any arthropathy. Synovial fluid 5NT activity in this group was significantly lower than in pyrophosphate arthropathy synovial fluid ($p<0.001$) and did not differ significantly from the normal control group, as shown in figure 19.

7.3 Synovial fluid 5'nucleotidase activity of inflamed and non-inflamed knees.

To assess the effects of disease activity or the clinical inflammatory state of the joint on synovial fluid 5NT activity, knees were assessed as "active" or "inactive" prior to aspiration, as described in chapter 2.2. Synovial fluid 5NT activity was found to be increased in active compared to inactive knees in pyrophosphate arthropathy ($p<0.01$), and osteoarthritis ($p<0.05$). No such difference was apparent in rheumatoid synovial fluid between active and inactive groups, as shown in figure 20.

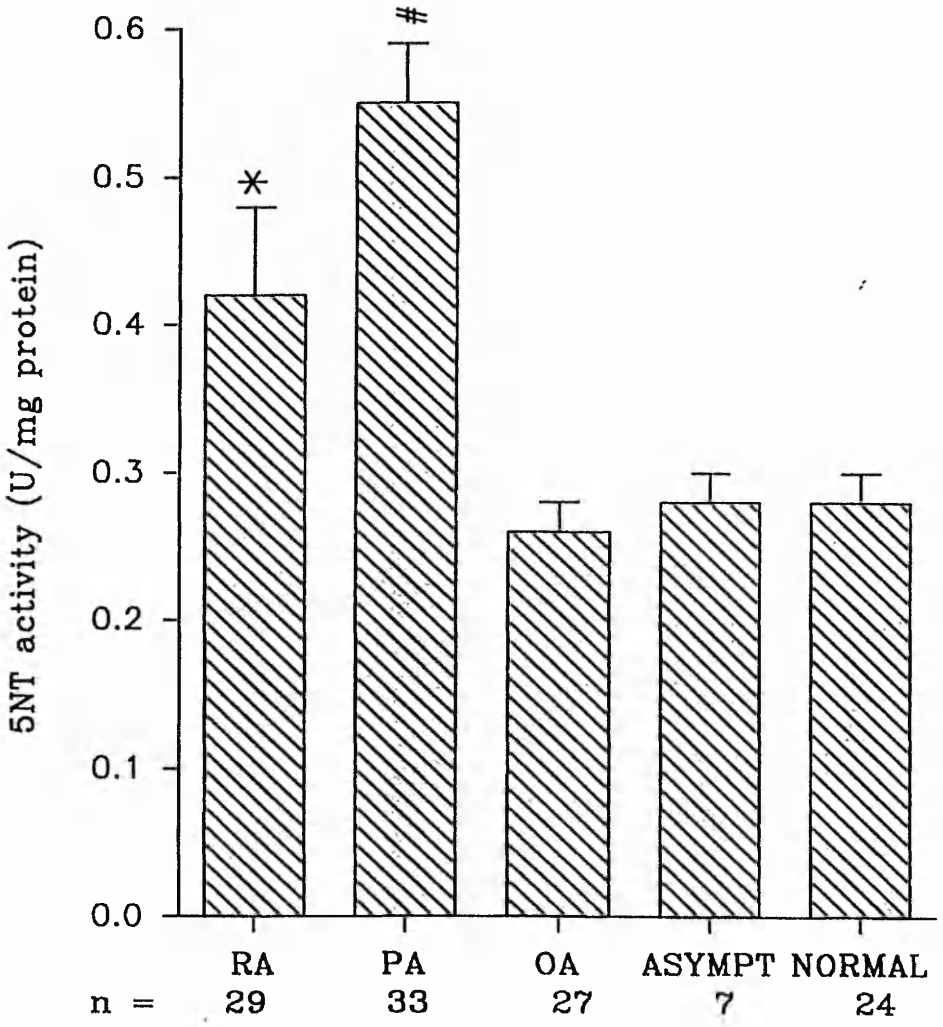


Figure 19: Synovial fluid 5NT activity of disease groups and normals. Results epresent the mean (±sem), n denotes subject number in each group. * synovial fluid 5NT activity was significantly greater in RA compared to OA ($p<0.001$) and normal ($p=0.02$). # synovial fluid 5NT activity was significantly greater in PA compared to RA ($p<0.05$); OA ($p<0.001$); ASYMPT and normal ($p<0.001$).

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis; ASYMPT = asymptomatic.

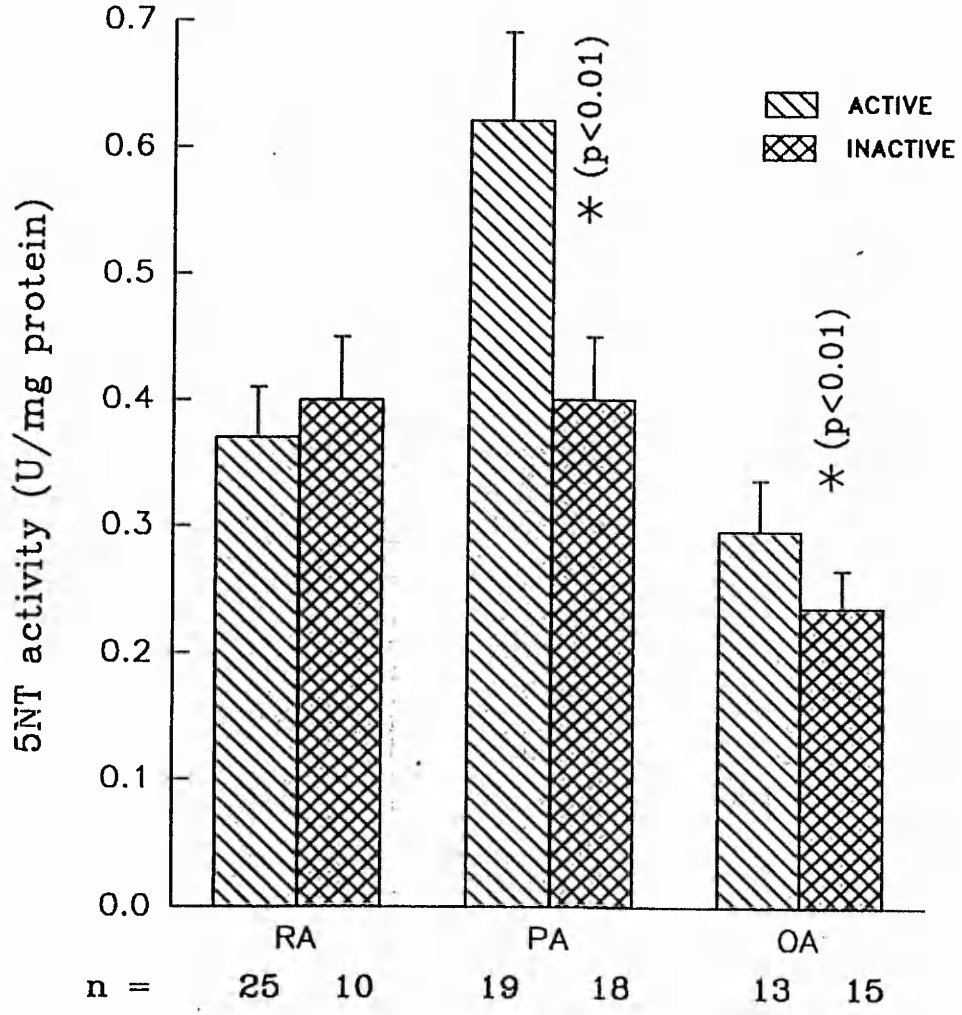


Figure 20: Synovial fluid 5NT activity in "active" and "inactive" knees of disease groups. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance between "active" and "inactive" knees, whilst p indicates the level of significance.

RA = rheumatoid arthritis; PA = pyrophosphate arthropathy; OA = osteoarthritis.

7.4 Discussion.

5NT activity has been reported as elevated in extracts of CPPD crystal containing cartilage compared to osteoarthritic cartilage extracts (Tenenbaum *et al* 1981) and in CPPD crystal containing synovial fluid compared to non-crystal containing synovial fluid (Rachow *et al* 1988, Wortmann *et al* 1991). In both instances a specific association between synovial fluid 5NT activity and CPPD crystal deposition was implied. Increased activity of articular cartilage 5NT could promote PPi generation by removal of AMP produced by pyrophosphohydrolysis of ATP. Consequently, an increase in PPi could then promote CPPD crystal deposition.

This study agrees with previous findings that 5NT activity is increased in synovial fluid in pyrophosphate arthropathy compared to that in osteoarthritis (Rachow *et al* 1988, Wortmann *et al* 1991) and normal. However, in contrast to Rachow and Wortmann's findings above, of low 5NT activity in non-crystal containing synovial fluids including rheumatoid arthritis, this study demonstrates a significant increase in 5NT activity in rheumatoid synovial fluid compared to osteoarthritic and normal. This may be due to differences in assay technique. High naturally occurring ADA in rheumatoid synovial fluid may have affected our results, as 5NT is measured indirectly by ammonia generated by ADA in the assay system.

Increased synovial fluid 5NT activity was also found in the clinically active groups of pyrophosphate arthropathy and osteoarthritis compared to the inactive groups,

suggesting a relationship with the clinical inflammatory state of the joint. 5NT activity was also measured in asymptomatic, crystal containing joints in the absence of inflammation and apparent disease. Activity in this group was found to be not significantly different to activity in normal synovial fluid. This finding together with the finding of increased 5NT activity in the active compared to inactive groups in pyrophosphate arthropathy and osteoarthritis and increased 5NT activity in rheumatoid synovial fluid compared to osteoarthritic and normal, suggests that synovial fluid 5NT activity, rather than reflecting CPPD crystal deposition in the cartilage, is a reflection of the inflammatory state of the joint.

Previous studies on rheumatoid synovial fluid have shown increased 5NT activity compared to osteoarthritis, and that the activity correlates with the inflammatory state of the joint (Henderson *et al* 1980, Farr *et al* 1973, Kendall 1979). The source of rheumatoid synovial fluid 5NT in these studies was located to the synovium.

The source of synovial fluid 5NT in this study, therefore, is more likely to be from the inflamed synovium as found in rheumatoid joints than from the articular cartilage itself, demonstrated by the normal levels of synovial fluid 5NT activity found in the asymptomatic joints. The increased activity of 5NT found in pyrophosphate arthropathy synovial fluid may be explained by the inflammatory element of CPPD crystals on the synovium, perhaps only reflected in the disease state.

This study, therefore, by the inclusion of normal and asymptomatic controls, demonstrates that synovial fluid 5NT activity is a more accurate indicator of the inflammatory state of the joint than of CPPD deposition in the articular cartilage. 5NT activity within the articular cartilage matrix as described by Tenenbaum *et al* (1981) may be a more accurate indicator of any relationship with CPPD deposition, than that found in synovial fluid.

CHAPTER 8: Synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity in metabolic diseases predisposing to calcium pyrophosphate dihydrate crystal deposition.

8.1 Introduction.

To study the putative association between CPPD crystal deposition and certain metabolic diseases, knee synovial fluid PPi was measured in patients with untreated metabolic disease and in normal controls, to determine whether a localised elevation of this ion exists, promoting CPPD crystal deposition within the joint. In addition, the activity of synovial fluid NTPP was measured in order to assess the role of this enzyme in the production of articular PPi in metabolic disease.

Urinary PPi was also measured in each metabolic disease group to ascertain whether a systemic aberration of PPi metabolism was apparent.

Five metabolic disease groups were studied: primary hyperparathyroidism, idiopathic haemochromatosis, hypomagnesaemia, adult-onset hypophosphatasia and primary hypothyroidism. The diagnostic criteria for the metabolic disease groups are described in detail in chapter 2.3. Since synovial fluid PPi concentrations are elevated in knees with pyrophosphate arthropathy, only synovial fluid from subjects with asymptomatic, non-arthritic knees were used in this study.

Clinical Group	(n)	Synovial Fluid			Urine			Knee CC *
		N° tested for PPi	N° tested for NTPP	N° with CPPD crystals	Number tested	PPi/Creatinine # (mean ± sem)		
Hyperparathyroidism	(21)	9	2	2	14	2.1	(0.4)	3
Haemchromatosis	(5)	6	4	3	5	2.6	(0.7)	2
Hypomagnesemia	(2)	2	2	1	2	0.6	(0.2)	2
Hypophosphatasia	(5)	0	0	-	5	5.0	(1.0)	3
Hypothyroidism	(27)	11	9	1	20	2.1	(0.6)	0
Normal controls	(50)	50	50	0	20	2.4	(0.4)	0

µM/mM
* Chondrocalcinosis

Table 4: Synovial fluid sample numbers and urinary PPi levels in subjects with metabolic diseases and in normal control subjects

Due to the small volume of synovial fluid obtained from asymptomatic knees, or exclusion through presence of knee symptoms or structural arthropathy of these patients, plus difficulties obtaining fresh, fasting urine samples, synovial fluid PPi and NTPP activity and urinary PPi were measurable only on a limited number in each group. CPPD crystals were identified in some synovial fluid samples (with or without accompanying chondrocalcinosis) in each disease group. Since the joints from which these crystal containing fluids came from were asymptomatic, these synovial fluid samples were included in the study. Final sample numbers along with presence or absence of chondrocalcinosis and CPPD crystals are described in table 4.

8.2 Synovial fluid inorganic pyrophosphate concentrations of metabolic disease groups.

A significant increase in synovial fluid PPi compared to normal was observed in the hyperparathyroid group ($p<0.05$) and in the haemochromatotic group ($p<0.05$) as shown in figure 20. Increased synovial fluid PPi was also detected in the hypomagnesemic group but sample numbers were too small to make a valid statistical comparison to the normal group in this case. In contrast, the hypothyroid group showed significantly reduced synovial fluid PPi compared to normal ($p<0.05$).

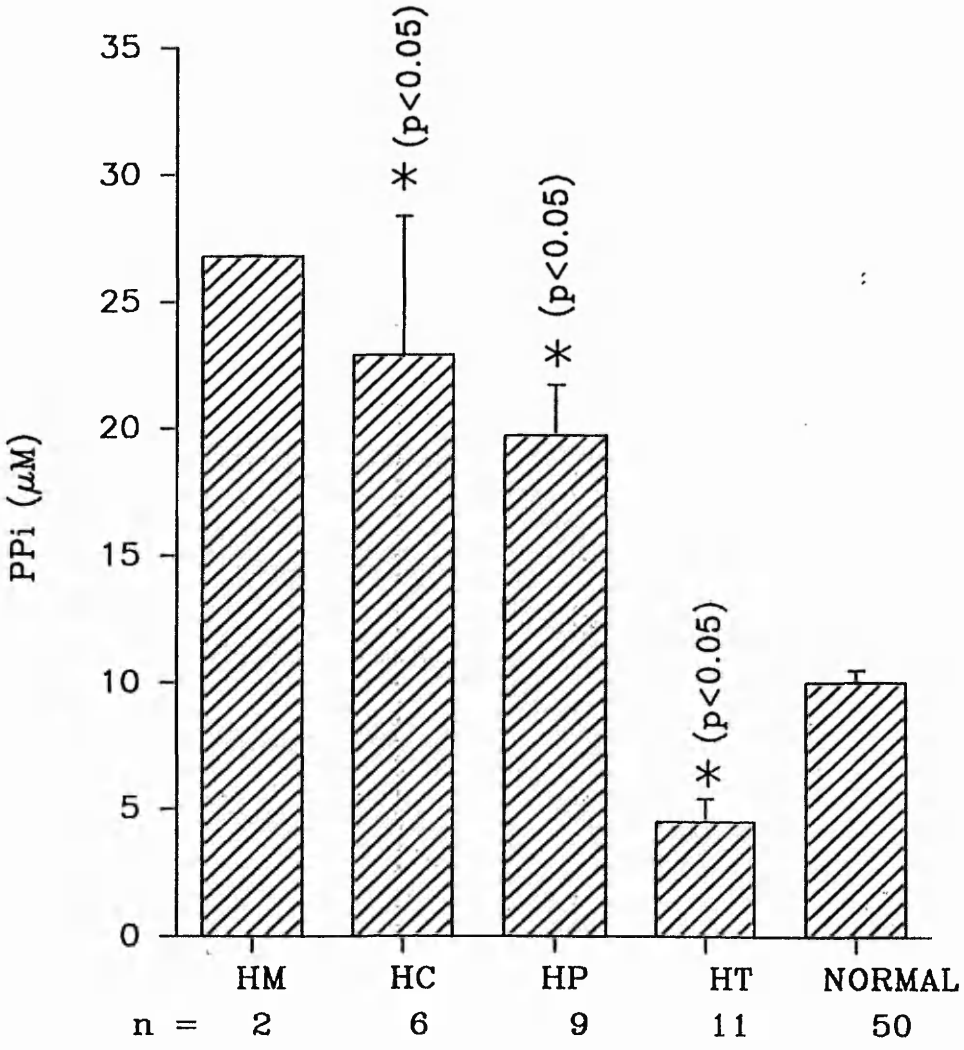


Figure 21: Synovial fluid PPi levels of metabolic disease groups and normals. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to normal, whilst p indicates the level of significance.

HM = hypomagnesaemia; HC = haemochromatosis; HP = hyperparathyroidism; HT = hypothyroidism.

8.3 Synovial fluid nucleoside triphosphate pyrophosphatase activity of metabolic disease groups.

A concurrent increase in synovial fluid NTPP activity with PPi concentration was found in the haemochromatosis group, where NTPP activity was significantly higher than normal ($p<0.001$), as shown in figure 22. A similar increase in NTPP activity was observed for the hypomagnesaemia group. As with PPi, however, sample numbers were too small to make a valid statistical comparison to normal. The hyperparathyroid group did not differ from normal for NTPP activity, despite the increase found in synovial fluid PPi. NTPP activity in the hypothyroid group, however, showed a concurrent decrease with PPi concentration compared to normal ($p<0.05$).

8.4 Urinary inorganic pyrophosphate concentrations of metabolic disease groups.

Urinary PPi was measured in all the metabolic disease groups and in normal controls. A significant increase in urinary PPi was found in the hypophosphatasia group compared to normal ($p<0.05$), as shown in table 4. All other groups did not differ significantly from the normal range.

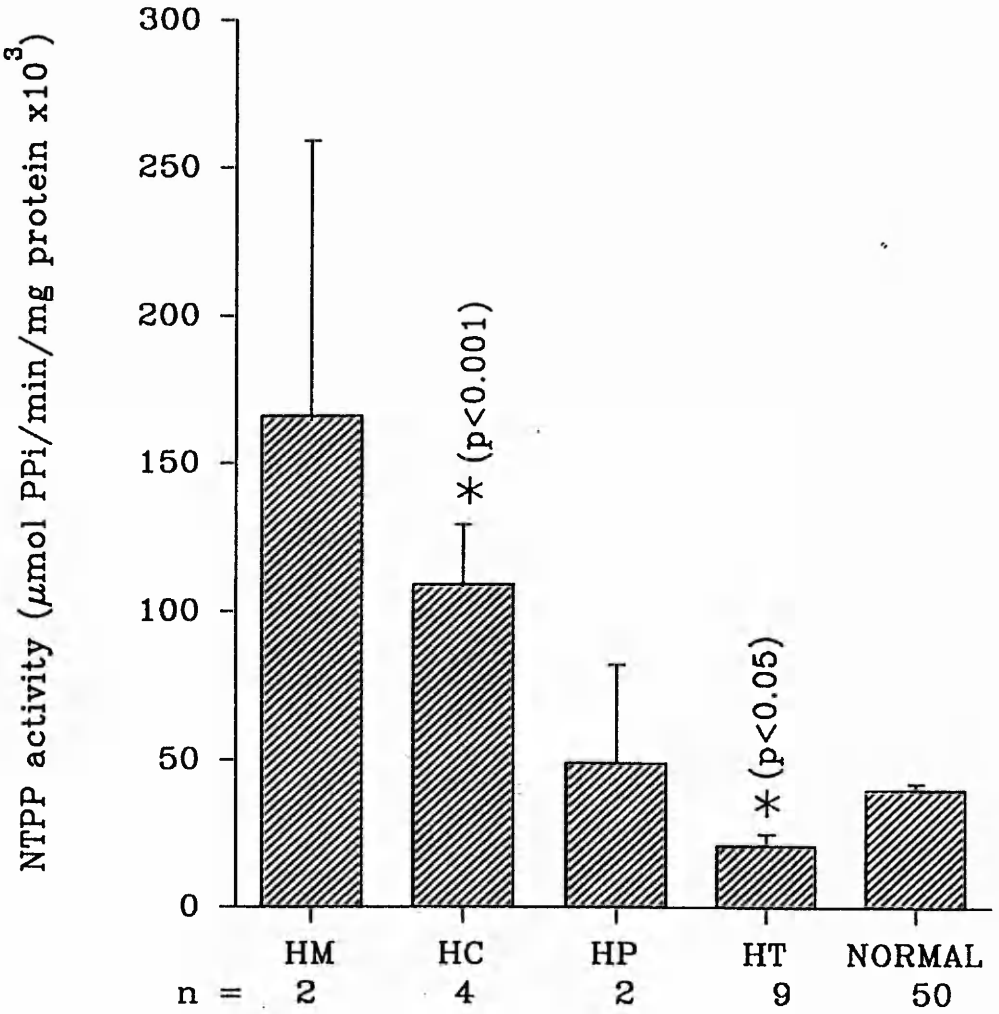


Figure 22: Synovial fluid NTPP activity of metabolic disease groups and normals. Results represent the mean (±sem) for each group, n denotes subject number in each group. * indicates statistical significance compared to normal, whilst p indicates the level of significance.

HM = hypomagnesaemia; HC = haemochromatosis; HP = hyperparathyroidism; HT = hypothyroidism

8.5 Discussion.

In the five metabolic diseases studied hypophosphatasia was the only one to associate with elevated urinary PPi levels. Increased urinary PPi in hypophosphatasia, an observation first reported by Russell (Russell *et al* 1965), demonstrates the systemic abnormality in PPi metabolism found in this disease, due to a deficiency in alkaline phosphatase. In hypothyroidism normal urinary PPi levels were apparent, consistent with the reported elevation of urinary PPi in the converse situation of hyperthyroidism (Russell and Hodgkinson 1969). Although elevated serum and urinary PPi levels have also been reported in osteogenesis imperfecta (Armstrong *et al* 1975), neither this disease nor hyperthyroidism were included in the study as no association has been determined between either diseases and CPPD crystal deposition.

The significant increase in synovial fluid PPi found in haemochromatosis, hypomagnesaemia and hyperparathyroidism, compared to normal controls suggests a localised disturbance of PPi metabolism within the joint. Sufficient volumes of synovial fluid were not obtained in hypophosphatasia for measurement of PPi or NTPP activity. Assuming a state of equilibrium between synovial fluid and cartilage (Camerlain *et al* 1975. Russell 1976, Rachow and Ryan 1988), this increased synovial fluid PPi is likely to reflect similar or even greater increases within the cartilage, increasing the ion product [PPi x Ca] and the promotion of CPPD crystal deposition (Russell 1976).

In the haemochromatotic group the concomitant increase in synovial fluid NTPP activity with PPi concentration suggests stimulation of enzyme activity in this disease state, which as a result could be responsible for the increased synovial fluid PPi. There is no previous evidence of iron stimulating NTPP activity, or of the effects of iron on chondrocyte metabolism in-vitro. McCarty *et al* (1970) demonstrated that erythrocyte intracellular pyrophosphatase was inhibited by ferrous ions. If this is also true for pyrophosphatases within cartilage, then the combined stimulation of NTPP together with decreased pyrophosphatase activity in haemochromatosis could lead to a significant increase in PPi within the joint.

Although both synovial fluid PPi levels and NTPP activity were elevated in the hypomagnesemic group, sample numbers were small and results must be interpreted cautiously. Previous in-vitro findings have shown that a reduction in magnesium does not affect chondrocyte NTPP activity (Caswell and Russell 1984). Although, in a study by Bischoff *et al* (1975) NTPP was shown to be magnesium dependent.

One favourable hypothesis for the increased synovial fluid PPi in the hypomagnesaemia group is decreased activity of ALP and other magnesium dependent pyrophosphatases. A systemic deficiency of pyrophosphatase activity was not evident in this study, as demonstrated by normal urinary PPi levels. However, localised effects on pyrophosphatases within cartilage cannot be ruled out. Although these data suggest that increased NTPP activity within the joint is

a putative mechanism which could promote increased PPi, in the hypomagnesemic group, small sample numbers and the evidence that NTPP is magnesium dependent do not allow any valid conclusions to be drawn.

Normal synovial fluid NTPP activity in the hyperparathyroid group, despite increased PPi concentrations, concurs with previous findings that chondrocyte NTPP activity is not promoted by increasing ionic calcium (Caswell and Russell 1984), or by the addition of PTH to chondrocyte monolayers (Ryan *et al* 1989). Altered NTPP activity is therefore an unlikely explanation for the increased synovial fluid PPi found in this study.

A more indirect relationship may exist between hyperparathyroidism and increased synovial fluid PPi levels. For example, PTH has been shown to have a mitogenic effect on chondrocytes, in the presence of calcium ions and increased adenylate cyclase activity in the plasma membrane (Schluter *et al* 1989, Centralla *et al* 1989). This increased adenylate cyclase activity could result in a local increase in PPi (McGuire *et al* 1980(a), Tell *et al* 1973), though whether this PPi is leaked to the extra-cellular space is unknown. PTH also increases cAMP and glycosaminoglycan synthesis by chondrocytes (Enomoto *et al* 1989). A by-product of this is PPi, which may be exported with the matrix products and lead to increased extracellular PPi concentrations.

McCarty and colleagues (1970) proposed the inhibition of pyrophosphatases by

calcium ions. However, a systemic increase in PPi is not evident in hyperparathyroidism. Although elevated urinary levels were found by Avioli *et al* (1965, 1966), subsequent studies have failed to confirm this (Russell and Hodgkinson 1969, Lewis *et al* 1966) and plasma PPi has also been found to be normal (Russell *et al* 1971). In addition to increased synovial fluid PPi levels found in hyperparathyroidism, raised ionic calcium would also increase the ion product [Ca x PPi] promoting CPPD crystal deposition.

In contrast to haemochromatosis, hypomagnesaemia and hyperparathyroidism, the hypothyroid group showed decreased synovial fluid PPi and NTPP activity compared to normal. This finding concurs with a previous report suggesting that NTPP activity or PPi accumulation was neither stimulated nor increased on the addition of TSH to chondrocyte cultures (Ryan *et al* 1989). Thus the findings in the hypothyroid group are not indicative of an association between this disease and CPPD crystal deposition, as a result of increased PPi within the joint. Other recent studies have similarly suggested a lack of association between hypothyroid disease and CPPD crystal deposition (Komatireddy *et al* 1989, Smith 1990, Job-DesLandre *et al* 1993).

In addition to the effects on articular PPi metabolism by metabolic disease, demonstrated for the first time in this study, other effects on CPPD crystal deposition have previously been postulated. Cartilage matrix changes and iron salts may promote crystal nucleation in haemochromatosis (Schumacher 1976, Caswell

et al 1983, Hearn *et al* 1978) and increased calcium in hyperparathyroidism may increase the ion product [PPi x Ca] (Russell 1976). If such conditions exist within the cartilage matrix of these metabolic diseases, together with the increase of extracellular PPi concentration demonstrated by this study, favourable conditions for the promotion of CPPD crystal deposition would thus exist.

CHAPTER 9: Synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity in familial pyrophosphate arthropathy.

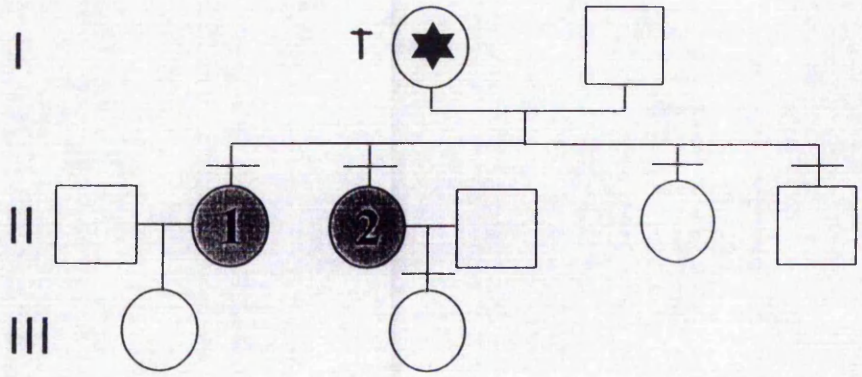
9.1 Patient characteristics.

In this first report of familial chondrocalcinosis in the UK, five affected families were studied in an effort to elucidate any abnormality of articular PPi metabolism leading to CPPD crystal deposition.

Of the five families included in this study, one originated from Belfast, Northern Ireland, where ancestors dated back at least as far as the early nineteenth century. The other four families had apparently dwelt in the Midlands for at least four preceding generations. Family names and anthropometric characteristics suggested nothing other than British ancestry. There was no evidence of consanguinity in any family. Family pedigrees are illustrated in figures 23 and 24. Clinical and radiographic details are described in Doherty *et al* (1991(b)). Twenty-nine of the 33 invited relatives agreed to participate in the study (11 male, 18 female).

Two of the five affected families were characterized by premature-onset polyarticular chondrocalcinosis with little associated structural arthropathy. Affected members of the other three families resembled sporadic disease in showing predominantly late-onset, oligoarticular chondrocalcinosis with mild arthritis and destructive change in only one case.

Family 1



Family 2

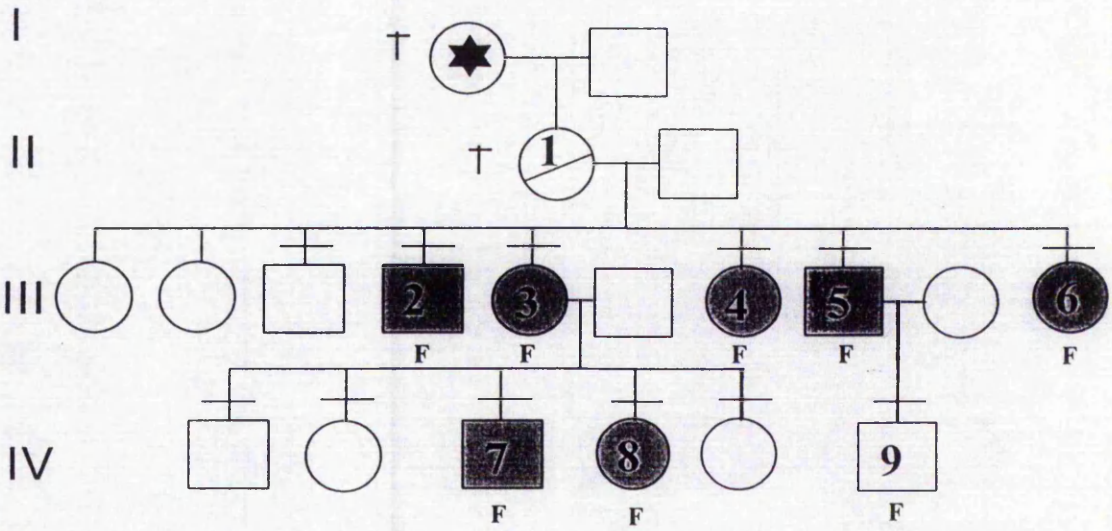
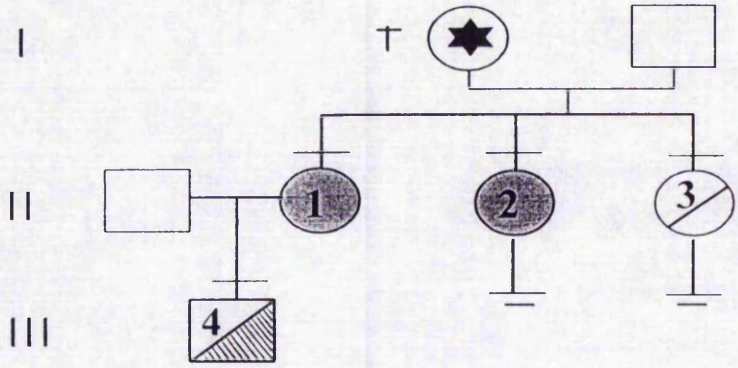
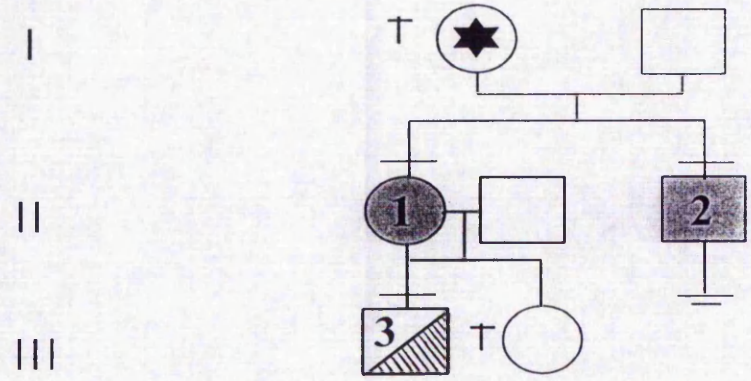


Figure 23: Pedigrees of families 1 and 2, with premature onset polyarticular chondrocalcinosis. Numbers I-IV represent generations (circles = women, squares = men). Examined subjects are indicated by horizontal bars, deceased subjects by an adjacent cross. Shading represents chondrocalcinosis + synovial fluid confirmation of CPPD crystals; a diagonal bar alone represents chondrocalcinosis without synovial fluid crystal confirmation. Stars signify deceased subjects with unconfirmed historical evidence of similar arthropathy. In family 2 "F" signifies occurrence of childhood fits.

Family 3



Family 4



Family 5

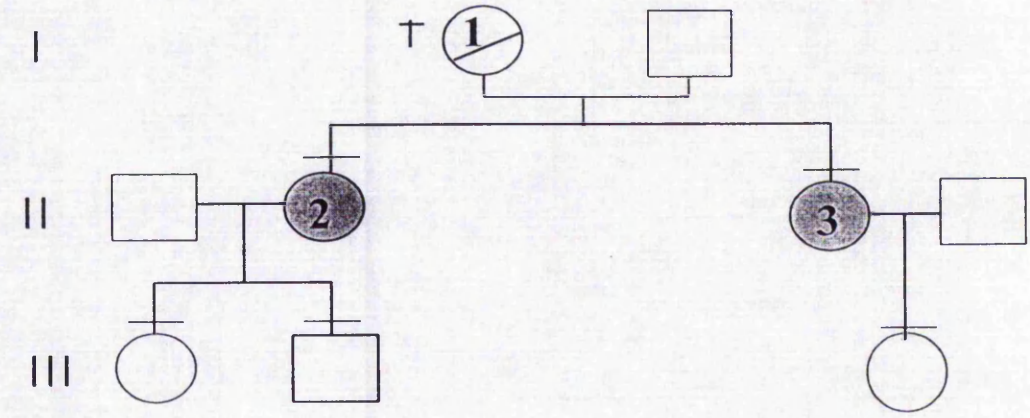


Figure 24: Pedigrees of families 3, 4 and 5. (double horizontal bars indicate married with no children; a diagonal bar with shading indicates synovial fluid CPPD crystal identification with no chondrocalcinosis; otherwise same key as in figure 23.

In the five families 15 subjects had radiographic chondrocalcinosis and confirmed synovial fluid CPPD crystals (definite pyrophosphate arthropathy); three had radiographic chondrocalcinosis without available synovial fluid confirmation; two had synovial fluid CPPD crystals without chondrocalcinosis; and in four deceased subjects there was a clear history of joint disease despite no investigative evidence. From 16 of the 29 subjects studied, sufficient synovial fluid was collected for the assay of PPi and NTPP activity. Assays of synovial fluid and urinary PPi and synovial fluid NTPP activity are described in chapter 3.3 to 3.5.

Details of the familial subjects together with the sporadic and normal controls are described in chapter 2.4.

9.2 Synovial fluid inorganic pyrophosphate and nucleoside triphosphate pyrophosphatase activity of familial compared to sporadic pyrophosphate arthropathy and normal subjects.

Previous studies on familial pyrophosphate arthropathy have implicated a generalised abnormality of PPi metabolism, expressed phenotipically only in cartilage, in CPPD crystal deposition (Lust *et al* 1981. Ryan *et al* 1986). However, as yet, there have been no studies of articular cartilage PPi metabolism in familial pyrophosphate arthropathy, to assess any localised abnormality within the joint.

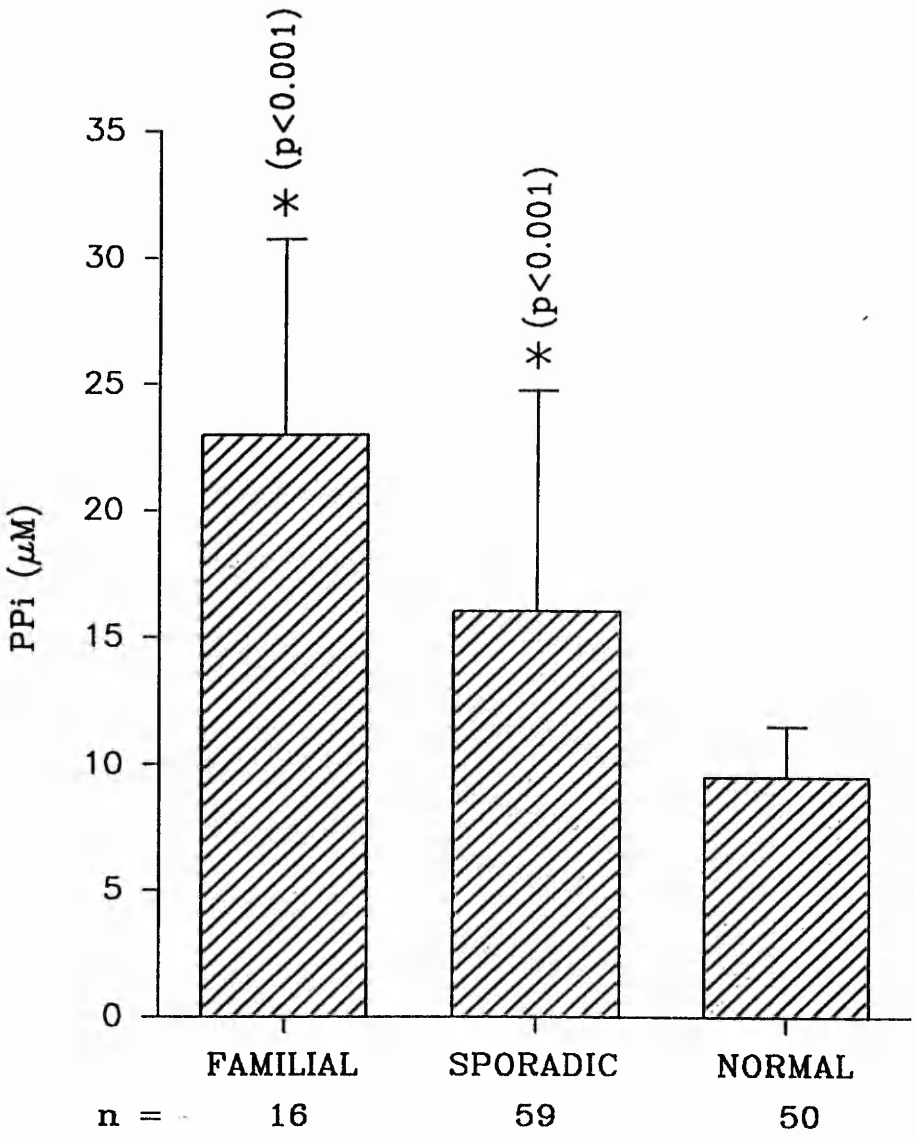


Figure 25: Synovial fluid PPi levels of familial pyrophosphate arthropathy, sporadic pyrophosphate arthropathy and normals. Results represent the median (± interquartile range) for each group, n denotes subject number in each group. * indicates statistical significance compared to normal, whilst p indicates the level of significance. No significant difference was observed between the familial and sporadic groups for synovial fluid PPi levels.

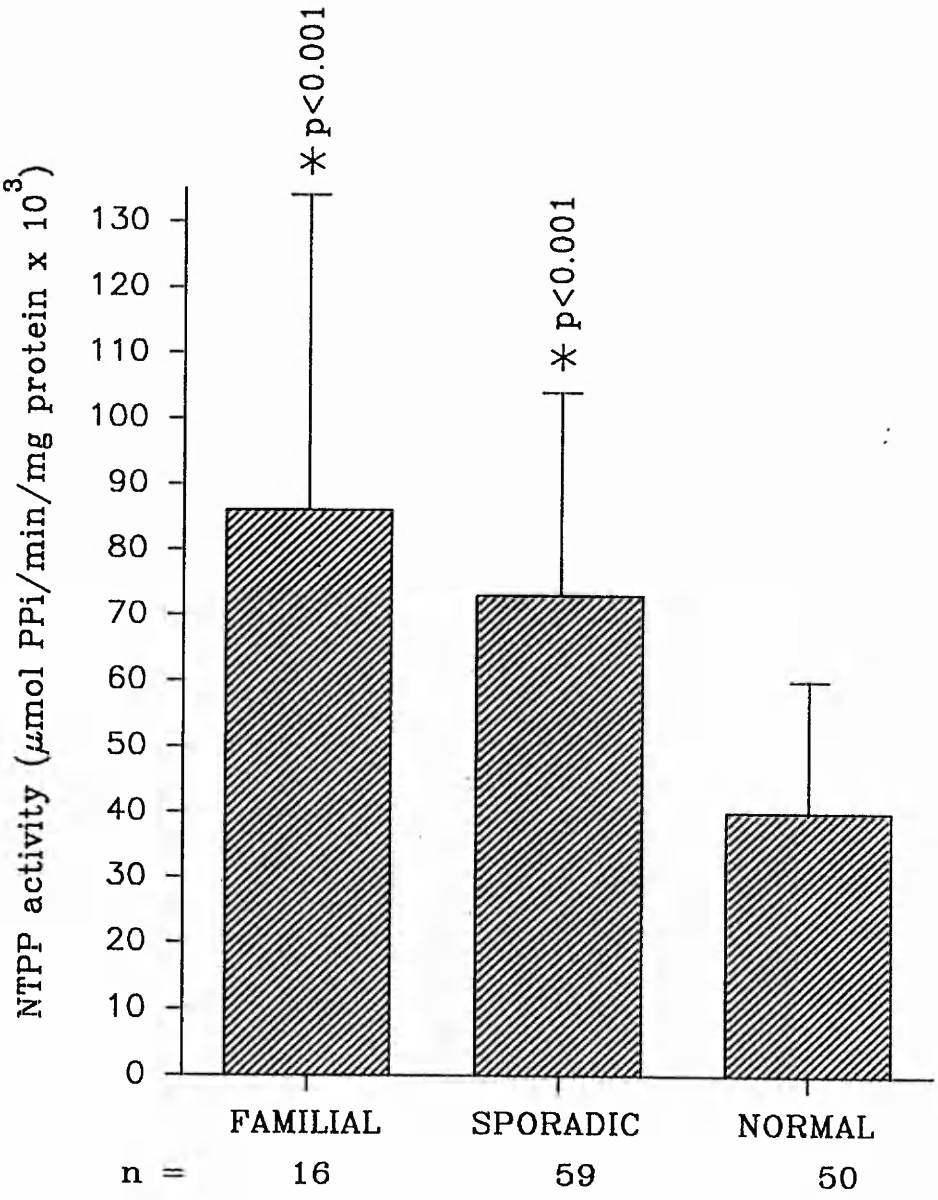


Figure 26: Synovial fluid NTPP activity of familial pyrophosphate arthropathy, sporadic pyrophosphate arthropathy and normals. Results represent the median (± interquartile range) for each group, n denotes subject number in each group. * indicates statistical significance between disease groups and normal, whilst p indicates the level of significance. No significant difference was apparent between familial and sporadic pyrophosphate arthropathy for synovial fluid NTPP activity.

In this study synovial fluid PPi levels and NTPP activity were measured in five affected families to assess any localised increase in production of PPi within the joint and to determine whether the activity of NTPP may be altered in familial pyrophosphate arthropathy compared to the sporadic form of the disease.

No significant difference in synovial fluid PPi levels or NTPP activity was observed between clinically uninflamed knees of familial and sporadic patients, as shown in figures 25 and 26. However, PPi levels and NTPP activity in both groups were significantly higher than in normal knee synovial fluid ($p<0.001$). There was no difference between familial pyrophosphate arthropathy patients and normal controls for urinary PPi corrected for creatinine (median, interquartile range: 1.24, 0.5-3.85 and 2.54, 0.5-3.81 μmol PPi/mmol creatinine respectively).

9.3 Discussion.

In the five families studied, two different phenotypes were observed as described previously. Two of the families were typical of the florid, easily recognised form with premature-onset acute attacks, polyarticular involvement and exuberant chondrocalcinosis with little associated structural arthropathy, similar to those families described in Holland (van de Korst 1966, van de Korst *et al* 1974, van de Korst and Geerards 1976), Sweden (Bjelle 1981, Bjelle 1982), Canada (Gaudreau *et al* 1981), and the USA (Moskowitz and Katz 1964, Richardson *et*

al 1983, Brem 1982). The other 3 families, however, were more typical of the late onset, pauciarticular form that is clinically and radiologically indistinguishable from sporadic pyrophosphate arthropathy (Rodriquez-Valverde *et al* 1980, Fernandez-Dapica *et al* 1986, Rodriguez-Valverde *et al* 1988. Riestra *et al* 1988). Although the numbers are small and radiographs of all adult family members was not possible, the inheritance patterns are consistent with autosomal dominant transmission as illustrated in figures 25 and 26.

The synovial fluid PPi and NTPP results suggest that although the common abnormality of increased PPi and NTPP activity seems to be shared by both the familial and the sporadic groups, the florid polyarticular familial form of the disease does not seem to be due to increased chondrocyte NTPP activity alone, as reflected by synovial fluid measurement. It may be that undetected familial cases exist within the sporadic group which could result in artificial elevation of synovial fluid NTPP activity and PPi levels. This could particularly occur with the late onset, pauciarticular form of the disease which is clinically and radiologically indistinguishable from sporadic pyrophosphate arthropathy. However, there was no obvious difference in synovial fluid PPi or NTPP activity between patients with florid, dense polyarticular CPPD deposition and those with modest, limited crystal deposition.

Normal urinary PPi in the familial group does not support a systemic abnormality of PPi metabolism. Similar synovial fluid findings in the sporadic and familial

groups agree with previous reports of a localised abnormality within the joint (McCarty 1975, Caswell *et al* 1983, Rachow and Ryan 1988, Pattrick *et al* 1991). However, a generalized abnormality of PPi metabolism has been implicated in familial CPPD crystal deposition. Lust *et al* (1981), found increased intracellular PPi levels in cultured skin fibroblasts and lymphocytes from affected family members of a French kindred compared to non-affected members and unrelated normal controls. Ryan *et al* (1986) found elevated intracellular PPi levels in skin fibroblasts but normal NTPP activity of patients with familial pyrophosphate arthropathy. They also found increased PPi in fibroblasts from patients with sporadic pyrophosphate arthropathy, together with increased NTPP activity. Both studies were hampered, however, by the considerable overlap found between normal and familial intracellular PPi levels.

The mechanism for increased intracellular PPi levels in these cell types is uncertain. No excretion or external accumulation of PPi from fibroblast or lymphoblast cultures was found (Lust *et al* 1981), in contrast to the finding in chondrocyte cultures, where the extra-cellular build up of PPi is thought to contribute to crystal deposition. Thus, if PPi is not exported, or is rapidly hydrolysed in fibroblasts and lymphoblast cultures, this may explain the normal urinary PPi levels found in this study. Alternatively cartilage matrix factors that influence crystal nucleation and growth may be under genetic influence. Bjelle (1981) suggested that a metabolic disturbance of cartilage matrix may be responsible for CPPD crystal deposition in familial pyrophosphate arthropathy. He

found that hydroxyproline levels were low and keratin sulphate levels were increased in familial cartilage compared to normal controls and that such an abnormality, together with increased PPi within the joint could promote CPPD crystal deposition. Additionally lipoproteins which promote CPPD crystal deposition may also be an inherited factor in familial pyrophosphate arthropathy (Ishikawa *et al* 1989).

This study, by measuring synovial fluid PPi and NTPP activity in familial pyrophosphate arthropathy for the first time, suggests that a similar abnormality of PPi metabolism exists in both the sporadic and familial forms of the disease. However, additional factors under genetic influence which affect CPPD crystal deposition may also exist, being the inherited factor in affected families.

CHAPTER 10: Nucleoside triphosphate pyrophosphatase activity of human articular cartilage in organ culture: a comparison between disease groups and in subjects with unilateral chondrocalcinosis

10.1 Introduction.

The aim of the following study was to assess whether altered chondrocyte PPi metabolism reflects a generalised abnormality of cartilage in predisposed individuals or a localised response to joint damage in pyrophosphate arthropathy. The objectives of the study were to compare chondrocyte NTPP activity between involved and uninvolved knees from cadavers with unilateral chondrocalcinosis and/or pyrophosphate arthropathy. Also to compare chondrocyte NTPP activity between the disease groups pyrophosphate arthropathy, osteoarthritis, rheumatoid arthritis and normal joints.

10.2 Nucleoside triphosphate pyrophosphatase activity of articular cartilage from involved and uninvolved knees of cadavers with unilateral chondrocalcinosis and/or pyrophosphate arthropathy.

The number of pairs of knees from cadavers was unfortunately very limited. Only one pair from a single cadaver was obtained throughout the study, in which chondrocalcinosis and/or pyrophosphate arthropathy was absent.

10.3 Nucleoside triphosphate pyrophosphatase activity of articular cartilage in disease groups and normals.

As with the cadaveric cartilage samples, cartilage from subjects undergoing knee replacement surgery were also limited in numbers, preventing any comparison between groups. Moreover, results of NTPP activity and PPi elaboration from cartilage containing CPPD crystals were vastly elevated by dissolution of the crystals into the incubation media. Only 2 samples of pyrophosphate arthropathy cartilage were obtained and two rheumatoid samples, no osteoarthritic samples were obtained.

10.4 Discussion.

Since chondrocyte NTPP activity is thought to be a major source of articular PPi, this ecto-enzyme has been implicated in the pathogenesis of pyrophosphate arthropathy (Rachow and Ryan 1988). The aim of this study was to determine whether a generalised abnormality of PPi metabolism, expressed as increased chondrocyte NTPP activity, may exist in subjects with pyrophosphate arthropathy.

This study was hampered, however, by inadequate sample numbers, from both the cadaveric and the replacement surgery sources. In addition, measurement of chondrocyte NTPP activity and PPi elaboration from CPPD crystal containing

cartilage in organ culture was impracticable due to the dissolution of CPPD crystals, resulting in vastly elevated PPi levels in the culture media. If the chondrocytes were subsequently grown in monolayer culture, this would still not warrant the effective removal of all crystals from the cell cultures, ensuring accurate results.

Ryan *et al* (1981) and Caswell *et al* (1983) have likewise, reported the dissolution of CPPD crystals into the culture media in a similar experiment. Other studies, however, have not reported vastly elevated PPi levels in culture media of chondrocalcinotic cartilage in organ culture (Tenenbaum *et al* 1981, Howell *et al* 1984). However, these studies employed different methods of measuring NTPP activity using radioactive labelled ATP as the substrate, and measuring the labelled products AMP and PPi, avoiding the measurement of any unlabelled PPi from crystal dissolution. The greatly elevated media PPi concentrations may also have an affect on ecto-NTPP activity, however, for example by product inhibition.

In light of the results from this study and the other studies mentioned above, NTPP activity in chondrocalcinotic cartilage in organ or monolayer culture cannot be ascertained, unless a method guaranteeing the exclusion of PPi produced as a result of CPPD crystal dissolution is employed. Also studies on the effect of increased PPi concentrations on NTPP activity need to be investigated, to ensure accurate results in the presence of CPPD crystal dissolution. As samples were not available in this study these methods could not unfortunately be applied.

CHAPTER 11: General discussion.

Substantial evidence now associates increased articular PPi with CPPD crystal deposition in pyrophosphate arthropathy. The proposed mechanisms by which increased articular PPi may result are varied (Rachow and Ryan 1988). However, this study like others, favours the increased activity of the chondrocyte ecto-enzyme NTPP. The synovial fluid PPi and NTPP findings in this study parallel *in-vitro* studies on cartilage; synovial fluid reflecting conditions within the cartilage matrix.

Studies on normal human synovial fluid have been very limited previously (Altman *et al* 1973(a)). This study by including a large normal control group for the first time, has established a normal range for synovial fluid PPi concentration and the enzyme activities studied. A clearer distinction between normal and diseased levels, and an accurate indication of where true increases in enzyme activities exist have been established

The findings of increased synovial fluid PPi concentration and NTPP activity in pyrophosphate arthropathy compared to osteoarthritis and rheumatoid arthritis, confirms previous findings (McCarty *et al* 1971, Altman *et al* 1973(a), Silcox *et al* 1974), and the positive correlation of synovial fluid PPi and NTPP activity verifies the role of NTPP as a principle producer of synovial fluid PPi. The normal synovial fluid PPi levels and NTPP activity found in osteoarthritis supports the

association between an error in PPi metabolism and CPPD crystal deposition in pyrophosphate arthropathy.

The increased synovial fluid PPi and NTPP findings in pyrophosphate arthropathy, therefore, may relate to the hypertrophic nature of the disease, associating elevated PPi and NTPP activity to increased cellularity and biosynthesis. By contrast in rheumatoid arthritis the low synovial fluid PPi levels and NTPP activity may reflect the atrophic nature of this disease and negative correlation with CPPD crystal deposition (Doherty *et al* 1984).

The effects of drug treatment on articular PPi metabolism in any of the diseases studied here, has not been reported previously. Due to the contrasting treatments of arthritic diseases, especially between osteoarthritis and pyrophosphate arthropathy compared to rheumatoid arthritis, and even within these disease groups, this area of study would prove very difficult to pursue. Many patients in this study were examined and had their knees aspirated prior to treatment, including all of the metabolic and many of the familial patients.

Reduced synovial fluid PPi levels in rheumatoid arthritis have been attributed to the effects of inflammation on synovial flow and faster clearance of PPi from the joint (Camerlain *et al* 1988). Similarly low concentrations of synovial fluid PPi in acute pseudogout compared to chronic pyrophosphate arthropathy (Silcox and McCarty 1973) suggest an association between inflammation and decreased PPi

concentrations.

The relationship between the clinical inflammatory state of the joint and synovial fluid PPi levels and NTPP activity has not previously been investigated. In this study, by assessing knee inflammation preceding synovial fluid aspiration, it was possible to determine effects on PPi within disease groups. The method of assessing knee inflammation in this study uses a summated score of six clinical features and has been shown to correlate with complement activation and synovial fluid white blood cell count in several arthritides (Doherty *et al* 1988, Hamilton *et al* 1990).

The results of this study suggest that clinical inflammation has effects on reducing synovial fluid PPi levels in pyrophosphate arthropathy and rheumatoid arthritis, but has no discernable affect in osteoarthritis. The lack of association in osteoarthritis between inflammation and PPi metabolism, as was similarly found in osteoarthritis between inflammation and complement activation products (Doherty *et al* 1988), suggests that different inflammatory mechanisms exist in uncomplicated osteoarthritis in comparison to pyrophosphate arthropathy, perhaps related to the inflammatory element of CPPD crystals.

As the inflammatory state of the joint significantly affects the outcome of synovial fluid studies on PPi metabolism, an assessment of clinical inflammation at the time of joint aspiration, therefore, is pertinent, in such synovial fluid studies.

For increased PPi levels to be achieved within articular cartilage, increased production, decreased removal or both need to exist. Already in this study, increased production has been demonstrated, as a result of increased activity of chondrocyte NTPP. Additional effects on PPi metabolism by increased activity of synovial fluid 5NT was not observed in this study, as previously proposed (Rachow *et al* 1988, Wortmann *et al* 1991). Increased ecto-5NT activity by catalysing the reaction: AMP → adenosine + phosphate would pull the reaction ATP → AMP + PPi in the direction of synthesis, thus favouring increased extracellular PPi. In terms of decreased removal of PPi, pyrophosphatase activity in the form of ALP activity was not deficient in pyrophosphate arthropathy synovial fluid in this study. Rather than exhibiting an association with CPPD crystal deposition as previously suggested, both synovial fluid 5NT and ALP activity demonstrated an association with joint inflammation in pyrophosphate arthropathy, the enzyme activity correlating with disease activity. The source of these synovial fluid enzymes has been located to the synovium (Farr *et al* 1973, Henderson *et al* 1980). Therefore, synovial fluid levels of 5NT and ALP activity are unlikely to reflect accurate enzyme activity within the articular cartilage, which are more relevant to conditions that associate with CPPD crystal deposition.

Very few studies have attempted to assess clinical inflammation. The data presented in this study suggests that the activities of the synovial fluid enzymes NTPP, ALP and 5NT and levels of PPi are all seen to be affected by disease activity. These data strongly support inclusion of such assessment in future

synovial fluid studies.

Studies on the effect of ageing on articular PPi metabolism is of interest, due to the prevalence of chondrocalcinosis or pyrophosphate arthropathy in the elderly (Ellman *et al* 1981). In the large group of normals studied, covering a broad age-range, no correlation of synovial fluid PPi or NTPP activity with age was apparent. Articular cartilage PPi metabolism would seem, therefore, not to be directly affected by ageing alone. The finding of increased synovial fluid PPi and NTPP activity in "normal" joints with CPPD crystals, however, does suggest that PPi metabolism is involved in CPPD crystal deposition. Additional factors, therefore, operating via effects on cartilage matrix, crystal nucleation and growth, may be relevant to age-associated CPPD crystal deposition.

The role of CPPD crystals in joint disease is uncertain, as chondrocalcinosis exists in asymptomatic joints. It was previously thought that the release of crystals into the synovial space would produce inflammatory episodes and cartilage damage promoting the disease process (Bennett *et al* 1975). However, evidence from this study suggests, that CPPD crystals exist in the synovial fluid as well as in the cartilage, in the absence of any symptoms. It is clear therefore that CPPD crystals do exist as "innocent bystanders". Any effect of CPPD crystals may depend on the presence of additional factors such as joint damage or disease.

The studies on metabolic abnormalities demonstrate for the first time evidence for

altered articular cartilage PPi metabolism in haemochromatosis, hypomagnesaemia and hyperparathyroidism. The increased synovial fluid PPi found in these diseases implicates an increase in the ion product [PPi x Ca] in the predisposition to CPPD deposition in these disorders. Furthermore limited data on haemochromatotic and hypomagnesemic synovial fluid suggest increased NTPP activity may be responsible for the elevated PPi levels found in these two diseases, whilst in hyperparathyroidism a more indirect relationship with PPi metabolism is suggested. No evidence for a systemic abnormality of PPi metabolism was apparent except for in hypophosphatasia.

The reduced synovial fluid levels of PPi and NTPP activity in hypothyroidism compared to normal, reflects the systemic reduction in metabolic activity in this condition. Furthermore, these reductions question the putative association between hypothyroidism and CPPD crystal deposition, at least due to an error in articular PPi metabolism.

In respect of familial pyrophosphate arthropathy, this study does not implicate an abnormality of articular PPi metabolism in familial predisposition. The findings of similar synovial fluid PPi levels and NTPP activity in familial and sporadic pyrophosphate arthropathy do not support a greater abnormality of PPi metabolism in the familial compared to the sporadic form of the disease. Normal urinary PPi levels in the familial group do not support a systemic abnormality of PPi metabolism as previously implied (Lust *et al* 1981, Ryan *et al* 1986). It is

possible, however, that urinary PPi levels do not reflect elevated intracellular PPi levels as found in these studies. Extracellular PPi is probably more relevant to CPPD crystal deposition, however, as CPPD crystals appear predominantly in perichondrocyte matrix (Caswell *et al* 1983), and PPi is thought unable to cross cell membranes (Felix and Fleisch 1977). The various cartilage matrix factors that influence crystal nucleation and growth may be under genetic influence, causing differences in crystal deposition between individuals.

It is evident that multiple factors play a role in familial CPPD crystal deposition, of which are under genetic influence is not known. The marked heterogeneity of familial pyrophosphate arthropathy, however, suggests that different mechanisms promoting CPPD crystal deposition may operate in different families.

The studies on NTPP activity in articular cartilage organ culture, unfortunately, were confounded by lack of sample numbers. Whether altered PPi metabolism is a generalised abnormality of articular cartilage in individuals with CPPD deposition or a localised response to joint damage would, however, prove interesting in future studies.

CHAPTER 12: Conclusions.

In this study, an error in articular PPi metabolism was evident as a mechanism promoting CPPD crystal deposition in pyrophosphate arthropathy. The chondrocyte ecto-enzyme NTPP is implicated in this error, having increased activity in pyrophosphate arthropathy compared to non-CPPD crystal arthropathies such as osteoarthritis and rheumatoid arthritis. Whilst articular NTPP is implicated in CPPD crystal deposition, the synovial fluid enzymes 5NT and ALP showed no such association, but did, however, relate to the inflammatory state of the joint. Similarly synovial fluid NTPP activity and PPi concentrations were also affected by clinical inflammation.

The effect of clinical inflammation on PPi metabolism varied between the disease groups studied, indicating that different inflammatory mechanisms exist in rheumatoid arthritis, osteoarthritis and pyrophosphate arthropathy. The inclusion of an assessment of clinical inflammation in future synovial fluid studies is strongly recommended.

Age, arthritic disease, metabolic disorders and familial tendency all show an association with CPPD deposition. Only in certain metabolic disorders is altered PPi metabolism implicated in this study: in haemochromatosis, hypomagnesaemia and hyperparathyroidism. NTPP is implicated as a source of the increased synovial fluid PPi in the former two conditions. In hyperparathyroidism, however, a more

indirect mechanism is proposed for increased articular PPi concentrations and predisposition to CPPD crystal deposition.

In familial pyrophosphate arthropathy, articular PPi metabolism did not appear to differ from the sporadic form of the disease, suggesting other mechanisms predisposing to CPPD crystal deposition may be under genetic influence. The synovial fluid studies on normal subjects indicate that ageing alone does not affect articular PPi metabolism. Other factors must exist in ageing cartilage promoting crystal deposition.

This study, by including a comprehensive group of normal controls, allowed a realistic comparison of synovial fluid PPi concentrations and NTPP activity between disease groups. It was thus demonstrated that in osteoarthritis, which is thought to predispose to CPPD deposition, PPi levels and NTPP activity did not differ from normal.

Whilst the synovial fluid studies ascertain an error in PPi metabolism exists in joints affected by CPPD deposition in sporadic pyrophosphate arthropathy,it is not known whether this error may be systemic in articular cartilage, also affecting asymptomatic joints. By comparing NTPP activity and PPi elaboration in chondrocyte cultures from subjects with unilateral chondrocalcinosis, this may be discernable. Due to insufficient sample numbers, however, it was not possible to pursue this part of the study to any conclusion. This subject would however, prove

an interesting area of future study.

It is evident, therefore, that mechanisms leading to the deposition of CPPD crystals are multifactorial, and in all the conditions that predispose to CPPD deposition, altered articular PPi metabolism alone cannot be implicated.

APPENDIX 1: List of suppliers

Amersham International Plc,
Amersham Laboratories,
White Lion Road,
Amersham,
Buckinghamshire.

BDH Laboratory Supplies,
Merck Ltd,
Hunter Boulevard,
Lutterworth,
Leicestershire.
LE17 4XN.

Boehringer-Mannheim UK,
(Diagnostics & Biochemicals) Ltd,
Bell Lane,
Lewes,
East Sussex.
BN7 1LG.

Calbiochem Novabiochem (UK) Ltd,
Unit C2A,
Boulevard Industrial Park,
Beeston,
Nottingham.
NG9 2JR.

Du Pont (UK) Ltd,
Diagnostics & Biotechnology Systems.
Wedgwood Way,
Stevenage,
Hertfordshire.
SG1 4QN.

Gibco Ltd,
PO Box 35,
Washington Road,
Abbotsinch Industrial Estate,
Paisley.
PA3 4EP.

Sigma Chemical Company Ltd,
Fancy Road,
Poole,
Dorset,
BH17 7BR.

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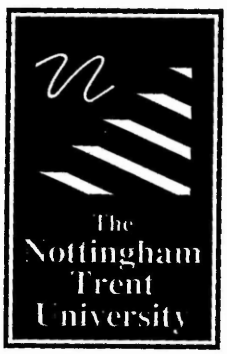

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Crystal deposition in hypophosphatasia: a reappraisal

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SUMMARY Six subjects (three female, three male; age range 38–85 years) with adult onset hypophosphatasia are described. Three presented atypically with calcific periarthritis (due to apatite) in the absence of osteopenia; two had classical presentation with osteopenic fracture; and one was the asymptomatic father of one of the patients with calcific periarthritis. All three subjects over age 70 had isolated polyarticular chondrocalcinosis due to calcium pyrophosphate dihydrate crystal deposition; four of the six had spinal hyperostosis, extensive in two (Forestier's disease). The apparent paradoxical association of hypophosphatasia with calcific periarthritis and spinal hyperostosis is discussed in relation to the known effects of inorganic pyrophosphate on apatite crystal nucleation and growth.

Hypophosphatasia is a rare inherited disorder characterised by low serum levels of alkaline phosphatase, raised urinary phosphoethanolamine excretion, and increased serum and urinary concentrations of inorganic pyrophosphate.[1–4] Clinical presentation classically takes one of three forms according to age: failure to thrive with high mortality in infancy, a condition resembling childhood rickets, or multiple fractures due to severe osteopenia in adults.[1 2] Disease severity is variable, especially in adult forms.[1 2] An association with premature, polyarticular chondrocalcinosis due to calcium pyrophosphate dihydrate (CPPD) crystal deposition is well described.[5–7]

Figure 1 outlines the suggested mechanisms for the osteopenia and chondrocalcinosis characteristic of this disorder. The major biological source of inorganic pyrophosphate (PPi) derives from pyrophosphorolysis of nucleoside triphosphates and diphosphates during biosynthesis of most major cell constituents.[8] Because alkaline phosphatase is the principal pyrophosphatase that converts PPi to orthophosphate, deficiency results in raised plasma and urinary concentrations of PPi.[3 4 8 9] Inorganic pyrophosphate avidly binds to apatite crystals[8 10–14] and, if unhydrolysed, is inhibitory to apatite crystal nucleation and growth,[10–16] leading to poor mineralisation and predisposition to fracture. Persistent increase of PPi, in addition, increases the calcium × PPi ionic product, predisposing to enhanced CPPD crystal deposition in cartilage.

Paradoxical presentation with calcific periarthritis—that is, excess apatite, in three adults with biochemical hypophosphatasia but no osteopenia prompted us to re-examine these postulates. Findings in these cases are contrasted with those in two elderly patients with more classic hypophosphatasia presenting with severe osteopenia and multiple fractures, and the asymptomatic father of one of the patients with calcific periarthritis.

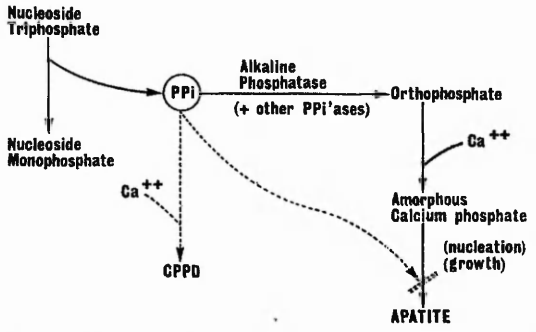

Fig. 1 *Simplified scheme showing putative mechanisms linking hypophosphatasia with both osteopenia and calcium pyrophosphate dihydrate (CPPD) crystal deposition: lack of alkaline phosphatase results in increased inorganic pyrophosphate (PPi), which then inhibits apatite formation and growth and also predisposes to CPPD crystal formation.*

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Patients and methods

Table 1 summarises the principal clinical, biochemical, and radiographic features of the six subjects. Each had consistently low serum alkaline phosphatase levels on repeat sampling: a metabolic screen, including serum urea, electrolytes, calcium, phosphate, magnesium, and thyroid function, failed to identify additional abnormality in any patient. Urinary phosphoethanolamine was estimated by high performance liquid chromatography after the method of Turnell and Cooper[17] with quantification by standard amino acid analysis (expressed as mmol phosphoethanolamine/mmol creatinine) and was increased on at least one occasion in 4/5 subjects investigated. The urinary PPi (μmol)/creatinine (mmol) ratio was measured by a modification of the technique described by McGuire *et al*[17] and was raised in all instances. Plain radiographs of hands, feet, knees, pelvis, and thoracic spine were obtained in all patients. The clinical histories are summarised in the following case reports.

CASE 1

A previously well 38 year old woman presented with acute onset of bilateral rotator cuff symptoms. Radiographs showed bilateral dense supraspinatus tendon calcification; needle aspiration of the left shoulder deposit showed material positive on ali red staining,[19] confirmed as hydroxyapatit infrared spectroscopy. Her inflammatory symp slowly settled on treatment with naproxen and steroid injection, but she persists with unal radiographic calcification and symptoms sugge subacromial mechanical impingement.

CASE 2

A previously fit 47 year old woman presented acute dactylitis of the left fourth toe, which res spontaneously over seven days. A radiograph 2) showed ill defined soft tissue calcification a site (absent on a radiograph repeated six m later); screening disclosed additional well de calcific deposits close to the greater trochante both hips, periarticular calcification at one hip dense nummular calcification in one supraspi tendon. Calcification in the annuli of several was noted and confirmed by computed tomogr scan. In the thoracic spine hyperostotic cha typical of those reported in limited Fores disease[20] were present at multiple levels. Sh had no further acute inflammatory episodes to

CASE 3

A previously fit 52 year old visiting Polish presented with acute pain, swelling, and eryt

Table 1 *Clinical, biochemical, and radiographic features of the subjects*

	Patient No					
	1	*2*	*3*	*4*	*5*	*6*
Sex, age	F, 38	F, 47	M, 52	M, 72	F, 78	M, 85
Clinical features						
Calcific periarthritis	+	+	+			
Fractures				+	+	
Acute pseudogout				+		
Biochemical features						
Mean (n=2–5) serum alkaline phosphatase (U/l)*	73	60	60	48	31	40
Max urine PEA† (mmol)/ creatinine (mmol)*	Not detected	0·046	No urine obtained	0·047	0·068	0·018
Max urine PPi† (μmol)/ creatinine (mmol)*	14	60		56	79	11
Radiographic features						
Periarticular calcification	+	+	+			
Chondrocalcinosis				+	+	+
Spinal hyperostosis/ Forestier's		+	+	+		+‡
Osteopenia				+	+	

*Normal values: serum alkaline phosphatase 80–280 U/l; urine phosphoethanolamine (mmol)/creatinine (mmol) ratio <0·010; PPi (μmol)/creatinine (mmol) ratio 0–5.
†PEA=phosphoethanolamine; PPi=inorganic pyrophosphate.
‡Plus diffuse idiopathic skeletal hyperostosis (DISH).

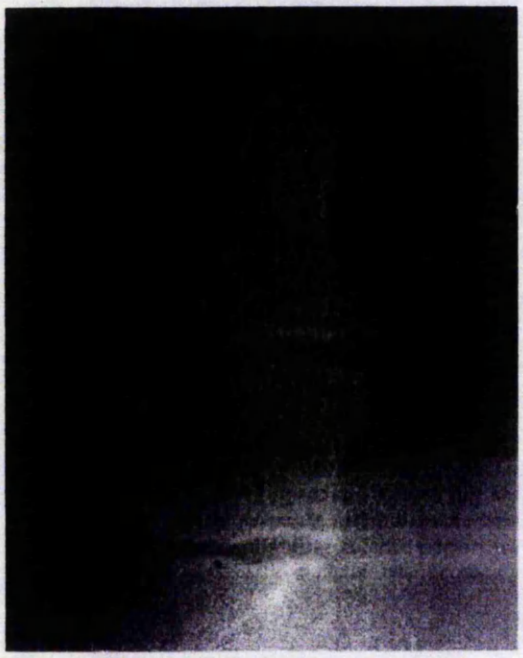

Fig. 2 *Left fourth toe radiograph (case 2) showing ill defined calcification, absent on a repeat radiograph six months later.*

around the left wrist. The radiograph showed discrete nummular calcification overlying the left carpus (Fig. 3), and needle aspiration showed calcific material with a spectrum on infrared spectroscopy consistent with carbonated hydroxyapatite. His acute inflammation settled on treatment with non-steroidal anti-inflammatory drugs alone, and a repeat wrist radiograph at 10 days, just before his return to Poland, showed considerable dispersal of the calcification (Fig. 3). Other radiographs showed normal mineralisation with no evidence of peripheral joint chondrocalcinosis or arthropathy. In the thoracic spine, however, marked calcification within the nucleus pulposus was present together with hypertrophic bone changes at multiple levels in a pattern consistent with limited Forestier's disease (Fig. 4).

CASE 4

A 72 year old man gave a 16 year history of multiple stress fractures and recurrent acute attacks of knee synovitis. The radiographic survey showed generalised osteopenia, multiple healed fractures, florid changes of Forestier's disease together with concave end plate depression in the thoracic spine, and polyarticular chondrocalcinosis of fibrocartilage and hyaline cartilage (knees, wrist triangular ligaments,

Fig. 3 *Lateral left wrist radiograph (case 3) showing discrete calcification (left), which had largely dispersed on the repeat film 10 days later (right).*

symphysis pubis) with no arthropathic change. Iliac crest bone biopsy showed widened osteoid seams; aspiration of both knees disclosed numerous crystals with morphology and polarised light characteristics of CPPD (no definitive crystal identification performed).

CASE 5

A 78 year old woman gave a 28 year history of multiple stress fractures. An iliac crest bone biopsy had shown features of osteomalacia, but her condition had proved resistant to vitamin D. Her radiographs showed severe osteopenia with old fractures and polyarticular chondrocalcinosis of hyaline and fibrocartilage (knees, wrist) but no changes of arthropathy. Synovial fluid from one asymptomatic knee showed CPPD crystals (confirmed by infrared spectroscopy).

CASE 6

This 85 year old man was the father of patient 3. He had experienced no locomotor symptoms but on investigation was found to have hypophosphatasia, extensive radiographic chondrocalcinosis (knees only) without arthropathy, normal mineralisation, and florid changes of Forestier's disease (Fig. 4): in addition, he showed ossification at the site of several entheses (ischial spines, acetabular labra, patella and Achilles tendon insertions, plantar fascia insertions) typical of diffuse idiopathic skeletal hyperostosis (DISH). Examination of small volumes of synovial fluid aspirated from both asymptomatic knees showed positively birefringent rhomboid crystals, confirmed as CPPD by Fourier transform infrared spectroscopy.

Discussion

Hypophosphatasia in these cases was diagnosed by consistently low serum levels of alkaline phosphatase (all cases), increased urinary phosphoethanolamine excretion (on at least one occasion in four of

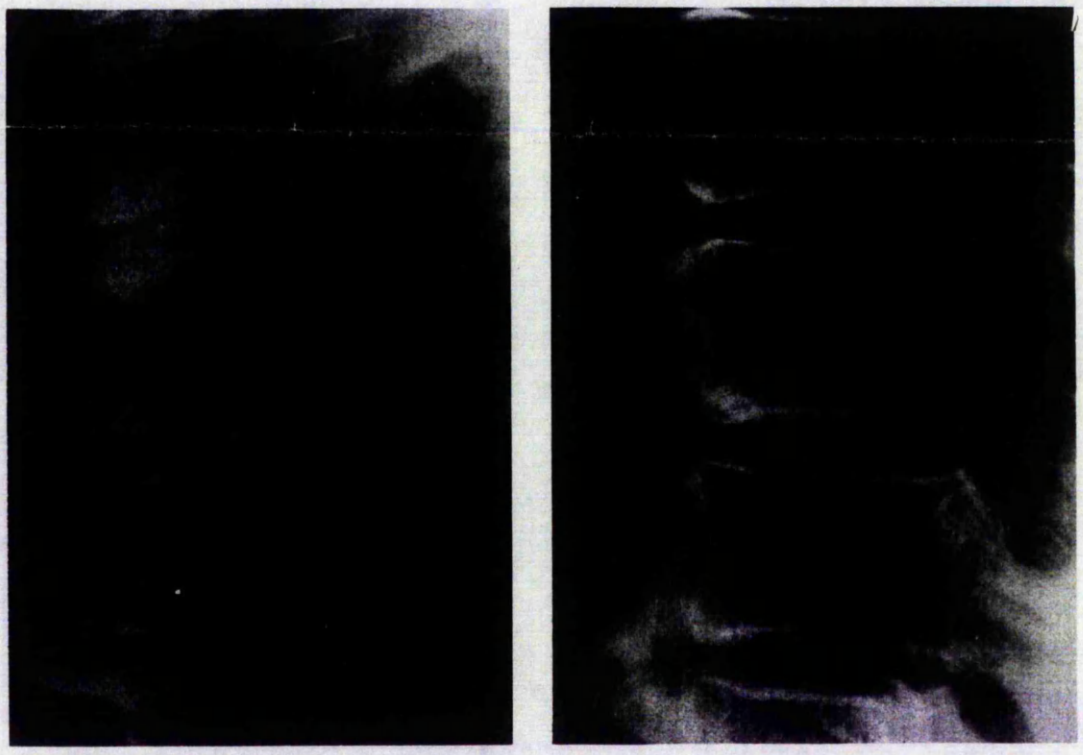

Fig. 4 *Lateral thoracic spine radiographs of case 3 (left) and case 6 (right). In the younger patient (case 3) there is intervertebral disc calcification (nucleus pulposus), normal intervertebral height, and anterolateral hypertrophic changes at multiple levels; in the older subject (case 6) there is normal intervertebral height and similar but more pronounced new bone formation typical of Forestier's disease.*

five subjects tested), and raised urinary PPi (in five cases tested). Interestingly, phosphoethanolamine excretion, usually regarded as mandatory for diagnosing hypophosphatasia, was variably positive in our subjects,[21] whereas urinary PPi excretion was consistently increased.[3] Although two patients had classic adult presentation with osteopenic fracture,[1 2] three had calcific periarthritis, an association noted in just two previous reports.[22 23] Although CPPD crystals may deposit in extracartilaginous sites,[24–27] diagnosis of apatite associated periarthritis was by clinical and radiographic characteristics — for example, nummular not linear tendon calcification,[24] with definitive crystal identification in two cases. Of further interest was the occurrence of early or established features of spinal hyperostosis (Forestier's disease) in four subjects. Spinal new bone formation in hypophosphatasia is well recognised, but a pattern typical of Forestier's disease has been emphasised in only one report.[22]

Although severe osteopenia is the expected consequence of hypophosphatasia, calcific periarthritis with normal bone density in three of our patients suggests that in certain situations low alkaline phosphatase levels may associate with stimulation rather than inhibition of apatite formation or growth. In vitro study of calcification mechanisms in epiphyseal growth cartilage[10 12 13 15 16] shows that at high concentration PPi is a potent inhibitor of apatite: adsorption of PPi onto the amorphous calcium/phosphate complex prevents transformation to crystalline hydroxyapatite, and adsorption onto apatite crystals inhibits aggregation and growth.[8 10–16] At lower concentrations, however, PPi promotes apatite crystal transformation and growth.[10 12 15 16] Inorganic pyrophosphate is conspicuously present in mineralising tissues and is thought by its dual effect to be a physiological regulator of matrix vesicle calcification.[15] Although osteoarticular concentrations were not assessed, it is conceivable that tissue PPi concentrations were in the range to stimulate apatite nucleation and growth in the three patients with calcific periarthritis (ectopic apatite), normal bone density, and modestly low serum alkaline phosphatase (mean (SD) 64·3 (7·5) U/l), whereas in those with osteopenia (insufficient apatite) and lower alkaline phosphatase levels (39·5 (12) U/l) tissue PPi was sufficiently raised to be inhibitory.

It is uncertain whether the three patients with calcific periarthritis represent a discrete mild subset of hypophosphatasia or the early development phase of classic osteopenic disease. Although periarticular calcification is often asymptomatic, and conceivably may previously have been present in the older subjects, lack of osteopenia in the father (case 6) of patient 2 supports a less severe variety. Serial alkaline phosphatase levels show variation in patients with hypophosphatasia, however, and it is possible that PPi concentrations in individuals may fluctuate from inhibitory to stimulatory. If spinal hyperostosis also reflects PPi related stimulation of mineralisation, such temporal fluctuation may explain concurrence of osteopenia and Forestier's disease in some cases. Spinal changes in two of our younger patients were typical of Forestier's disease,[28 29] but were not sufficiently extensive to fulfil Resnick's criteria.[30] The development of Forestier's disease remains unclear, but it seems likely that such changes represent early features of more florid, typical disease[20] seen in the two older subjects. Although four had axial hyperostosis, only one (case 6) had extraspinal new bone at the site of several entheses typical of diffuse idiopathic skeletal hyperostosis,[29 30] again supporting a milder variant of hypophosphatasia in those without osteopenia.

In addition to its effects on apatite, PPi itself may be incorporated as crystalline CPPD ($Ca_2P_2O_7.2H_2O$). Unlike apatite, CPPD crystal formation is mainly restricted to collagenous locomotor tissues (fibrocartilage and hyaline cartilage, tendon, capsule), and is particularly influenced by unidentified tissue factors that accompany aging.[8 31 32] All three patients over age 70 had chondrocalcinosis with synovial fluid CPPD crystal confirmation: this could reflect either the independent predisposing effect of aging, or the duration and extent of increased tissue PPi. Interestingly, only one had joint symptoms (acute pseudogout), and none had radiographic features of arthropathy. A similar clinicoradiographic pattern (asymptomatic or acute attacks, with isolated radiographic chondrocalcinosis) occurs in three other metabolic diseases predisposing to CPPD crystal deposition—namely, hyperparathyroidism,[33 34] hypomagnesaemia,[35 36] and Wilson's disease.[37] Such similarity supports predisposition by effects on PPi metabolism—for example, inhibition of alkaline phosphatase by calcium, cupric, and ferrous ions[38]; reduction in magnesium as co-factor for alkaline phosphatase[39]; stimulation of adenylate cyclase by parathyroid hormone.[8 39] By contrast, in haemochromatosis (another predisposing metabolic disease), although isolated chondrocalcinosis may occur, chronic symptoms with arthropathic change (cartilage/bone attrition, prominent cysts; distinctive distribution; often no overt synovitis) are more usual,[40 41] suggesting that other non-PPi related mechanisms may be involved in predisposition to chondrocalcinosis and arthropathy. Further study of diseases showing isolated metabolic defects may give insight into both physiological and

pathological mechanisms of calcium crystal deposition.

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SYNOVIAL FLUID CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTALS AND ALIZARIN RED POSITIVITY: ANALYSIS OF 3000 SAMPLES

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SUMMARY

Three thousand synovial fluids (1312 patients: chronic pyrophosphate arthropathy (CPA), 41%; osteoarthritis (OA), 12%; rheumatoid arthritis (RA), 16%) were examined for crystals, including calcium pyrophosphate dihydrate (CPPD), by polarized microscopy (score 0–3); calcific particles, by alizarin red positivity (ARP; 0–3); and total cell count. For 1150 fluids, local joint inflammation was assessed as 'active' or 'inactive' using a summated score of six clinical variables. CPPD and ARP scores did not correlate, but each showed positive correlation with age ($P<0.01$, $P<0.02$ respectively). Pseudogout had the highest mean CPPD score ($P<0.001$); intermittent CPPD positivity (range 8–100%) was seen in serially aspirated CPA joints, and there was no difference in CPPD positivity or score between active and inactive CPA. ARP was most frequent in OA subsets (72% of CPA, 46% of OA, 31% of RA; $P<0.001$). ARP was more frequent in active than inactive OA ($P<0.05$) but showed no association with inflammation in CPA or RA. Cell counts were higher in RA and pseudogout compared to OA and CPA, and in active compared to inactive RA. No correlation was found between ARP or CPPD scores and cell count. Cholesterol crystals were uncommon (0.2%) and showed no disease or joint predilection. In arthritic joints, CPPD and calcific particles particularly associate with the OA process and ageing. CPPD may contribute to acute and other calcific particles to chronic inflammation in OA.

KEY WORDS: Basic calcium phosphate, Apatite, Cholesterol, Crystals, Synovial fluid, Inflammation, Osteoarthritis.

A VARIETY of chemically diverse crystalline particles have been identified in synovial fluid (SF) from arthritic joints [1]. Many such particles possess inflammatory properties *in vitro* [2, 3] and their presence in SF has been used to define diagnostic categories [1, 3, 4]. However, in addition to practical difficulties in crystal identification [1, 5, 6], the relationship between SF crystals and *in vivo* inflammation, and the specificity of crystals for certain disease states remain uncertain [3, 4]. Such uncertainty is particularly true for calcium phosphate crystals associated with osteoarthritis (OA) subsets, notably calcium pyrophosphate dihydrate (CPPD) (the marker for 'CPPD crystal deposition disease' or 'pyrophosphate arthropathy' [7, 8]), and partial carbonate-substituted hydroxyapatite and other basic calcium phosphates (present in large amounts in 'Milwaukee shoulder syndrome' or 'apatite associated destructive arthropathy' [9, 10]).

PATIENTS AND METHODS

One thousand three hundred and twelve patients (762 female, 550 male: mean age 65, range 12–98 years) attending the Rheumatology Unit, City Hospital were included in the study. The major diagnostic groups were chronic pyrophosphate arthropathy (CPA; 41%), 'uncomplicated' OA (12%), and rheumatoid arthritis (RA; 16%). Patients with uncomplicated OA had symptomatic seronegative arthropathy with radiographs showing cartilage loss plus subchondral sclerosis or osteophytes, or both. None had radiographic chondrocalcinosis, SF CPPD crystals or evidence of other primary joint disease. CPA was defined as persistent (>3 months) symptomatic arthropathy with SF CPPD crystals and radiographic features of OA (with or without chondrocalcinosis). Patients with pseudogout had typical self-limiting acute episodes of synovitis with SF CPPD crystals and no evidence of coexistent joint disease (e.g. sepsis). Patients with RA fulfilled American Rheumatism Association criteria for classic or definite disease [11]. The diagnosis of other conditions was similarly based on clinical presentation/distribution of arthropathy together with appropriate serological and radiographic characteristics.

One thousand one hundred and fifty of the SF samples from knees were further assessed as 'active' or 'inactive' according to clinical inflammation at time of aspiration. This global assessment was made using a summated score of six clinical variables (Table I), where 0–2 was regarded as 'inactive' and 4–6 as 'active' [12]. 'Intermediate' joints scoring three were not included in further analysis relating to inflammation.

Joint aspirates were examined fresh and unspun (within 3 hours maximum). Crystals were identified by their morphology and sign of birefringence under normal and compensated polarized light (× 400). Each was stained with alizarin red S at pH 4.2 [13] and examined for presence of calcium containing particulate aggregates under ordinary light (× 100). SF white cell count (× 10^9/l) was by Coulter counter.

Both crystal content and alizarin red positivity (ARP) were quantified using a scoring system of 0–3 where 0 = no crystals/particles present, 1 = >2 crystals/particles identified, 2 = >10 crystals/particles identified or crystals/particles present in most fields,

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TABLE I
KNEE ASSESSMENT FOR CLINICAL INFLAMMATION

	Inactive	Active
Individual parameters		
Increased warmth	Absent	Present
Effusion	Absent–mild/not tense	Moderate–marked/tense
Synovial thickening	Absent	Present
Joint line tenderness (0–3)	0–1	2–3
Early morning stiffness	<1 h	1 h or more
Inactivity stiffness	<15 min	15 min or more
Global assessment	<3 parameters active	4–6 parameters active

and 3 = crystals/particles easily identified in all fields [14].

A single trained, experienced observer (JH) analysed all samples obtained during the period 1986–87. A second, identically trained observer (EH) analysed all samples collected during 1987–88. Although observers were not necessarily blinded to the principal working diagnosis for each patient, all samples were treated and examined in identical fashion.

The chi-squared test was used to compare categorical variables, the Mann-Whitney *U*-test (MWU) was used to compare independent groups and Spearman's rank correlation (r) was used to test for association.

RESULTS

Frequency of ARP between disease groups (Table II)

Frequency of ARP was CPA: 72%, OA: 46% and RA: 31%, with significant differences between all groups ($P<0.001$).

Correlation with age

In CPA, CPPD crystal score demonstrated positive correlation with age ($r = 0.183$, $P = 0.005$, $n = 313$). A positive correlation was also observed for ARP score and age in CPA ($r = 0.27$, $P<0.001$, $n = 313$), in OA ($r = 0.30$, $P<0.001$, $n = 199$), and in RA ($r = 0.29$, $P<0.001$, $n = 170$). There was no direct correlation between CPPD and ARP scores, suggesting independent age associations.

Association with clinical inflammation (Table II)

In CPA there was no difference in CPPD crystal score between samples from active and inactive joints. However, a higher CPPD score was observed in acute pseudogout compared to active CPA ($P<0.001$). For ARP the only association with clinical inflammation was a greater frequency in active compared to inactive OA ($P<0.05$).

Leucocyte counts (Table II)

Cell counts were higher in pseudogout and RA compared to OA and CPA ($P<0.001$). In relation to clinical inflammation, a difference between active and inactive joints was only seen in RA ($P<0.001$). CPPD and ARP scores did not correlate with cell counts.

Intermittent CPPD crystal identification

Serial aspiration (more than two occasions) was performed in 127 CPA knees over a mean period of 12 months (range 1 day–32 months). CPPD crystal identification was intermittent in 61% of joints with positivity ranging in individual joints from 8 to 100% (mean 66%).

CPPD in RA

CPPD crystals were identified in four (2%) RA patients (0.8% of all RA samples). For the purposes of this study such patients were retained within the RA group (not CPA).

Cholesterol crystals

Of the 3000 samples, only seven (four RA, two CPA, one seronegative spondarthropathy) from six patients contained cholesterol crystals. Joints involved were five knees, one shoulder and one wrist.

DISCUSSION

Identification of particles in SF is fraught with practi-

TABLE II
PATIENT CHARACTERISTICS, CRYSTAL PRESENCE AND LEUCOCYTE COUNT BY DISEASE CATEGORY

	OA		Acute	CPA		RA	
	Active	Inactive	pseudogout	Active	Inactive	Active	Inactive
Patients							
n	199		13	313		170	
M:F	1:1.8		1:1.2	1:2.2		1:2	
Mean age, years (range)	68 (27–92)		73 (51–95)	74 (33–98)		61 (28–84)	
n	56	95	24	330	331	177	42
ARP							
Frequency (%)	69	48	63	77	79	27	40
Mean score (±SD)	1.5 (0.7)	1.5 (0.7)	1.4 (0.5)	1.7 (0.7)	1.7 (0.7)	1.3 (0.5)	1.7 (0.8)
CPPD mean score (±SD)			2.1 (0.7)	1.1 (1.0)	1.2 (1.0)	1	1
Leucocyte counts (mean ±SD) × 10^9/l	0.7 (0.7)	0.5 (0.5)	26.5 (23.0)	2.0 (4.9)	1.0 (2.0)	12.9 (14.0)	5.8 (6.0)

cal difficulties. Definitive physical or chemical analysis is problematic, for example in terms of expense, demands on technician time, frequent requirement for a high particle yield, and manipulation of samples (with possible post-aspiration artefacts) prior to analysis [15, 16]. No adequate method of crystal/particle quantification has been devised, and estimation of total SF volume from which the sample has been obtained is impossible in a routine clinical setting. In this study we elected to use compensated polarized microscopy [17] and standard calcium staining [13]. Such techniques permit examination of a large number of fresh, untampered samples (impractical with more definitive, less applicable methodology). Polarized light microscopy is the routine method for identifying large, birefringent crystals [16], and ARP at acidic pH correlates well with presence of smaller calcium phosphate crystals or aggregates (strong ARP particularly suggesting apatite [13]). The method of particle quantification was derived empirically. Reproducibility of a similar scoring system has been shown to depend on observer experience [18]. Due to the many difficulties inherent in SF particle identification, in this study SF analysis was performed by two trained and experienced observers (over sequential time periods) and a large number of samples were included to reduce the bias of inconsistencies relating to method or observer.

The finding of ARP in 72% of CPA (an OA subset), 46% of OA, but only 31% of RA samples suggests an association between ARP and the OA process. Although the higher frequency of ARP in CPA could reflect 'false positive' staining of small CPPD crystals, the lack of correlation between ARP and CPPD scores in this group suggests that ARP predominently identifies calcium crystals other than CPPD. Support for basic calcium phosphates (BCP) as likely candidates for such particles comes from the association between ARP and BCP identified by electron microscopy [13, 19], and the frequent concurrence of CPPD and BCP in osteoarthritic joints [19, 20].

The strong positive correlation between SF CPPD score and ageing accords with previous reports of increasing frequency of CPPD deposition with age [4, 7, 8, 20, 21]. The similar, but independent, positive correlation between ARP score and ageing demonstrated both in OA and CPA (i.e. 'mixed crystal deposition') is also consistent with previous reports relating to frequency of BCP identification [22, 23]. Such agreement with published data for both CPPD and ARP lends support to the validity of our particle scoring system. The increased frequency of ARP with age in RA fluids has not previously been reported. This finding is of interest, however, in suggesting that factors which accompany ageing *per se* predispose to BCP deposition, irrespective of the underlying disease process. The very low frequency of CPPD identification in RA fluids accords with previous findings of a negative association between RA and crystal deposition [24]. Numerous hypotheses have been advanced but the factors promoting or inhibiting crystal deposition remain unclear [25].

Few SF studies have considered variability of joint inflammation and have characterized according to diagnostic category alone. As with particle quantification, there is no generally accepted system of grading 'joint inflammation'. The method we employed takes into account six clinical features which may reasonably be expected to reflect predominantly inflammation rather than mechanical damage or periarticular lesions. Summation of these features was used with exclusion of joints intermediate between the two extremes of the scoring range. Further grading, other than 'active' or 'inactive', was not attempted. Characterization of knees using this system has shown good agreement with SF complement activation, as a laboratory marker of 'inflammation', in some diseases [12]. In the present study, similar agreement was reflected by the higher SF cell counts in active compared to inactive RA knees. Interestingly, as with complement activation, very high counts were seen in acute pseudogout but low counts occurred in CPA with no difference according to degree of inflammation. The CPPD score followed an identical pattern, with the highest mean score in pseudogout and lower, similar mean scores in active and inactive CPA. These data accord with McCarty's experience that CPPD crystals are more readily demonstrable in pseudogout than CPA [14, 26], and support the contention that CPPD crystals are primary pathogenic agents in pseudogout but play a lesser role (if any) in the inflammation associated with CPA [4, 12]. For ARP, the only association with inflammation was a higher frequency in active compared to inactive OA. Although this would appear to support previous suggestions of an inflammatory role for BCP in OA [27, 28], occurrence of similar mean ARP scores in active compared to inactive OA, CPA or RA knees argues against a major contribution by BCP to joint inflammation.

In knees which had been repeatedly aspirated, CPPD crystal identification proved to be intermittent in most cases, with only 38% of patients being positive for CPPD on every aspiration. It is known that centrifugation of total SF samples and examination by electron microscopy will increase sensitivity of crystal identification [29]. Although our finding may principally reflect sampling or observer error, the methods we employed are those used routinely to identify SF crystals. The validity of delineating chronic 'pyrophosphate arthropathy' within the OA spectrum on the basis of limited SF analysis, as generally practised, is therefore to be questioned.

SF cholesterol crystals have been reported infrequently [30–32]. In one study of 1945 fluids examined over a 4-year period, Riordan and Dieppe [33] found cholesterol crystals only in shoulder fluids from six patients with RA, raising the possibility of site and disease specificity. In our study we identified such crystals in only seven of 3000 samples. These were in a variety of arthropathies, with no specific joint predilection and were never in abundance. The reasons for such disparity are unclear, but they presumably reflect differences in patient population. Other authors

have reported cholesterol in a variety of situations [30–32] and the role of such crystals in joint disease is unknown.

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Meta.fig 2 – metabolic data

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SYNOVIAL FLUID (SF) 5'NUCLEOTIDASE (5NT) AND ALKALINE PHOSPHATASE ACTIVITY IN ARTHRITIC AND NORMAL JOINTS

Edith Hamilton, Carolyn Belcher, Martin Pattrick, Michael Doherty
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Previous reports of elevated 5NT and low ALP SF levels in pyrophos arthropathy (PA) have associated these enzymes with calcium pyrophosphate dihydrate (CPPD) crystal deposition, via effects on pyrophosphate levels.

We measured 5NT and ALP levels (U/L/mg protein) in knee SF fr (1) patients with rheumatoid arthritis (RA; n=29), PA (n=33), or osteoarthritis (OA;n=27); (2) normal subjects (n=24); and (3) asymptomatic subjects with knees containing SF CPPD crystals (±radiographic chondrocalcinosis) but no arthropathy (ASYMP;n=7). time of aspiration knee inflammation was assessed as "active" or "inactive" using six clinical parameters. 5NT levels (mean±sem) w higher in PA (0.55±0.04) and RA (0.42±0.06) compared to OA (0.26±0 normal (0.28±0.02) and ASYMP (0.28±0.02) ($p<0.01$): levels were similar in normal and ASYMP. ALP levels were highest in RA (2.3±0 but also increased in PA (2±0.4) compared to normal (0.95±0.1); (p 5NT levels were higher in "active" compared to "inactive" knees in ($p=0.001$) and OA ($p<0.05$): no such association was apparent for AL

This study suggests that elevated SF 5NT activity associates w clinical inflammation but not CPPD crystal deposition. Contrary t previous reports SF ALP is not deficient in PA. The relevance of SF enzyme levels to CPPD crystal deposition is therefore questione

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INORGANIC PYROPHOSPHATE IN METABOLIC DISEASES PREDISPOSING TO CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION

MICHAEL DOHERTY, ALEXIS CHUCK, DAVID HOSKING, and EDITH HAMILTON

Inorganic pyrophosphate (PP_i) levels were estimated by radiometric assay in urine and in synovial fluid (SF) from asymptomatic, nonarthritic knees of patients with untreated metabolic disease and normal controls. SF PP_i was significantly elevated in patients with hyperparathyroidism (mean ± SEM 19 ± 3 μM; n = 9), hemochromatosis (23 ± 5 μM; n = 6), and hypomagnesemia (27 ± 0.1 μM; n = 2) compared with normal subjects (10 ± 0.5 μM, n = 50), and was low in patients with hypothyroidism (4.2 ± 2.3 μM; n = 11) ($P < 0.05$ all comparisons). Urinary PP_i was elevated only in those with hypophosphatasia. Local elevation of ionic PP_i may be relevant to the mechanism of crystal formation in metabolic diseases predisposing to calcium pyrophosphate dihydrate (CPPD) crystal deposition. The finding of low SF PP_i levels in patients with hypothyroidism further questions the association between this condition and CPPD.

Factors recognized as predisposing to chondrocalcinosis due to calcium pyrophosphate dihydrate (CPPD) crystal deposition include aging (1–4), the osteoarthritis process (2–7). a familial predisposition (1,7–12), and associated metabolic disease (1,7,13). In respect to the latter, there is impressive eviden associating premature, often widespread, CPPD cry tal deposition with hyperparathyroidism (13–16), h mochromatosis (13,17–19), hypophosphatasia (13,2 22), and hypomagnesemia (23–29). A less certa association between oligoarticular chondrocalcinos and hypothyroidism or myxedema is also suggest (30,31).

The physicochemical mechanisms wherel such diverse metabolic diseases predispose to CPP crystal deposition remain unclear, though differi putative effects on inorganic pyrophosphate (PP_i) m tabolism have been emphasized (32,33) (Figure 1). F example, alkaline phosphatase is an important pyr phosphatase (32), and either its reduction (in h pophosphatasia) or its inhibition (by increased iron calcium in hemochromatosis and hyperparathyroi ism, or by low cofactor levels in hypomagnesemi could elevate extracellular levels of ionic PP_i (32–34 Similar elevations could result from increased produ tion via stimulation of adenylate cyclase by exce parathyroid hormone (PTH) and thyroid-stimulati hormone (TSH) (32). In addition to effects on P metabolism, other postulated mechanisms include i crease in ($Ca^{2+} \times PP_i$) ion product by elevated calciu levels in hyperparathyroidism (32), promotion of cry tal nucleation by increased iron and calcium ion co centrations (32,35,36), enhancement of nucleation a growth by changes in cartilage matrix in hypoth roidism (32), and inhibition of CPPD crystal dissol tion by low magnesium concentrations (32,35,36).

Although there is in vitro evidence that su ports some of these postulated mechanisms, the pri cipal in vivo evidence for metabolic diseases is t estimated plasma and urine levels of PP_i that ha

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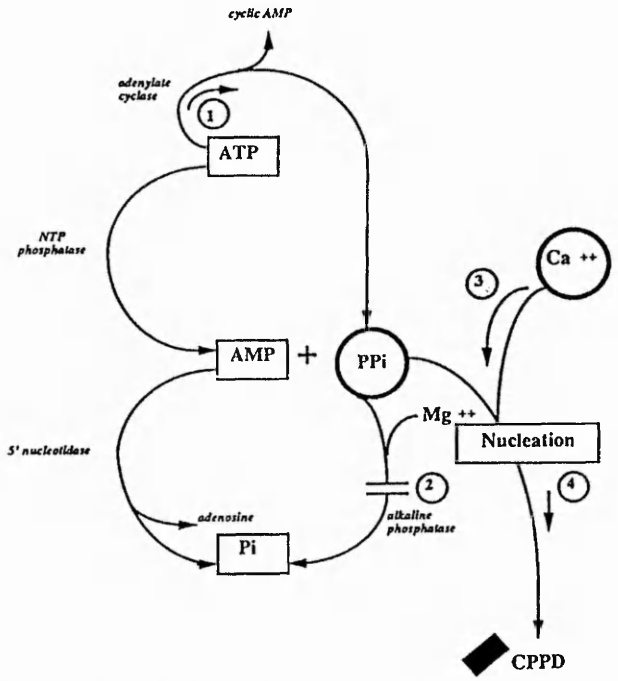

Figure 1. Theoretical sites of action on pyrophosphate metabolism by metabolic diseases predisposing to calcium pyrophosphate dihydrate (CPPD) crystal deposition: hyperparathyroidism (sites 1, 2, and 3), hemochromatosis (sites 2 and 4), hypophosphatasia (site 2), and hypomagnesemia (site 2). PPi = inorganic pyrophosphate; Pi = orthophosphate.

been described in just a few conditions (32,37–41). CPPD deposition is largely confined to intraarticular tissues, and local PP_i concentrations, particularly in a commonly involved joint (the knee), are therefore of greatest interest. We employed a sensitive and specific radiometric assay (42,43) to estimate PP_i levels in synovial fluid (SF) from the knees of patients with untreated metabolic disease and from normal controls. Since SF PP_i levels are elevated in knees with established pyrophosphate arthropathy (i.e., structural osteoarthritic changes [43–47]), we examined SF from only those subjects with asymptomatic, nonarthritic knees.

PATIENTS AND METHODS

Approval for this study was obtained from the local ethics committee.

Patient populations. Five disease groups were studied: primary hyperparathyroidism, idiopathic hemochromatosis, hypomagnesemia, adult-onset hypophosphatasia, and primary hypothyroidism.

Primary hyperparathyroidism. We studied 21 patients (12 women and 9 men, with a mean age of 72, age range 58–80), who had concurrent hypercalcemia and elevated serum PTH levels (mean 98 ng/liter, range 80–190, for intact PTH molecule, by immunoradiometric assay; normal range 10–55: Incstar Corporation, Stillwater, MN).

Idiopathic hemochromatosis. Five patients (3 men and 2 women, with a mean age of 63, age range 54–70), all with serum ferritin levels >3,000 μg/liter (normal 20–300) were studied. Liver biopsy was performed on 4 patients, each of whom had histologic changes consistent with idiopathic hemochromatosis. One patient had associated diabetes; 2 patients were siblings with familial disease.

Hypomagnesemia. Two female patients, ages 42 and 46, with persistent hypomagnesemia (mean serum levels 0.45 mmoles/liter, range 0.3–0.6; normal range 0.7–1.0) due to a congenital isolated renal tubular defect were studied. Neither had evidence of Bartter's syndrome.

Adult-onset hypophosphatasia. Five patients (3 women and 2 men, with a mean age of 64, age range 38–85) with persistent, low serum alkaline phosphatase activities (mean 50 IU/liter, range 31–69; normal range 80–280) and urinary excretion of phosphoethanolamine (mean 0.051, range 0.018–0.068 corrected to creatinine μmoles/mmole; normal absent) were studied. Two patients presented with recurrent fractures and osteopenia, 2 with calcific periarthritis, and 1 was the asymptomatic father of one of the patients (22).

Primary hypothyroidism. Twenty-seven patients (18 women and 9 men, with a mean age of 62, range 42–78), each with biochemical evidence of hypothyroidism and marked elevation of serum TSH levels (>50 mU/liter; normal range 0.1–4.3), were also studied.

At the time of study, all patients were untreated and had investigative evidence of current metabolic abnormality. None had clinical or biochemical evidence of other coexisting metabolic disease (blood screening included calcium, alkaline phosphatase, ferritin, magnesium, and thyroid function). Plain radiographs of knees (anteroposterior [AP] weight-bearing views and lateral views with the knee in 30° of flexion) were obtained in all patients to determine the presence of chondrocalcinosis and to exclude unsuspected structural arthropathy at this site.

Normal controls. Sixty-seven healthy adult volunteers (43 women and 24 men, with a mean age of 54, age range 22–83) with no clinical evidence of metabolic or joint disease served as controls. In those older than 50, knee radiographs (AP standing and lateral views) had been obtained to exclude occult chondrocalcinosis or structurally evident osteoarthritis (i.e., reduced interosseous distance and osteophyte formation). Medical undergraduates, hospital and laboratory staff, spouses of rheumatology patients, and members of the WRVS (Women's Royal Voluntary Service) comprised the majority of control subjects. (No financial reward was offered.) An isolated fluid "bulge" sign or isolated mild crepitus in older subjects (with normal radiographs) were not taken as reasons for exclusion.

Sample collections. SF was aspirated via a medial approach. Only asymptomatic, nonarthritic knees were aspirated. If no fluid or insufficient fluid was obtained from one knee, the opposite knee was then aspirated, provided that it, too, was asymptomatic and clinically normal, and that the

Table 1. Synovial fluid and urinary inorganic pyrophosphate (PP_i) levels in patients with metabolic diseases and in normal control subjects*

Clinical group (n)	Synovial fluid			Urine		Knee CC on radiographs
	PP_i (mean ± SEM μM)	No. tested	No. with CPPD crystals	PP_i/creatinine (mean ± SEM μmoles/mmole)	No. tested	
Hyperparathyroidism (21)	19 ± 3†	9	2	2.1 ± 0.4	14	3
Hemochromatosis (5)	23 ± 5†	6	3	2.6 ± 0.7	5	2
Hypomagnesemia (2)	27 ± 0.1†	2	1	0.6 ± 0.2	2	2
Hypophosphatasia (5)	–	0	–	5 ± 1†	5	3
Hypothyroidism (27)	4.2 ± 2.3†	11	1	2.1 ± 0.6	20	0
Normal controls (67)	10 ± 0.5	50	0	2.4 ± 0.4	20	0

* PP_i levels were measured as described in Patients and Methods. CPPD = calcium pyrophosphate dihydrate; CC = chondrocalcinosis.
† $P < 0.05$ versus normal controls: synovial fluid PP_i significantly lower in hypothyroidism group; all other comparisons significantly higher.

subject agreed. A sample of each SF was examined while fresh for the presence of CPPD crystals (by compensated polarized light microscopy). The remainder was collected into sterile plastic containers on ice, centrifuged at 2,500*g* for 15 minutes at 4°C, and the resulting supernatant was removed and stored at −20°C for PP_i assay. Second-void urine was collected in the morning, after overnight fasting. No additive was used, and the fresh samples were frozen to −20°C for PP_i assay.

PP_i assay. A modification (43) of a radiometric assay (42), involving conversion of PP_i to labeled 6-phosphogluconate was used to test for PP_i content. Duplicate 200-μl aliquots of SF (measured and transferred by positive-displacement pipette) or urine were extracted with 100 μl of trichloroacetic acid (TCA; 50% weight/volume). After addition of 800 μl of HEPES buffer (20 m*M*), samples were centrifuged at 2,500*g* for 15 minutes at 4°C. TCA was removed by addition of 1 volume of tri-n-octylamine in 3 volumes of 1,1,2-trichlorofluoroethane. Five hundred microliters of the resulting aqueous phase was neutralized by the addition of 100 μl of Tris/8 m*M* magnesium acetate buffer (pH 8). The final incubation mixture contained, in a volume of 140 μl, Tris (57 m*M*), magnesium acetate (5.2 m*M*), NADP (4 μM), glucose 1,6-diphosphate (18.6 μM), UDPG (7.5 μM), glucose-6-phosphate dehydrogenase (0.4 units), phosphoglucomutase (0.2 units), UDPG pyrophosphorylase (0.136 units), tritiated UDPG (0.08 μCi; specific activity 3–10 Ci/mmole), and 40 μl of sample or PP_i standard (0.5–8 μM).

The incubation reaction was performed in triplicate for 75 minutes at 37°C in a shaking water bath, then terminated by cooling on ice. A 2% activated-charcoal solution (deionized water; 250 μl) was added to separate any unreacted substrate from the labeled 6-phosphogluconate. After mixing, the samples were centrifuged for 15 minutes at 2,500*g* at 4°C. Then, 200 μl of supernatant was counted for radioactivity in a scintillation fluid.

To measure for hydrolysis of PP_i to orthophosphate (P_i) during incubation and extraction, we measured the tracer ^{32}P-PP_i that was added to the initial sample. P_i was removed by mixing 100 μl of each sample with an equal volume of ammonium molybdate (5% w/v) in HCl (2.7*M*). The resulting phosphomolybdate complex was then recovered into isobutanol/light petroleum (4:1 v/v). The aqueous phase was then counted for remaining ^{32}P-labeled PP_i in 0.1*M* HCl. Urinary PP_i estimations were corrected to creatinine (μmoles/mmole), measured colorimetrically at 550 nm using a Sigma (St. Louis, MO) diagnostic kit.

Statistical analysis. Differences between groups were compared by Mann-Whitney U test. Test for association was by Spearman's rank correlation.

RESULTS

Due to difficulties with obtaining fresh, fasting second-void urine samples (particularly from older patients and controls) or sufficient volumes of SF for full analysis, or because of exclusion through the presence of knee symptoms or structural arthropathy in patients with metabolic diseases, the final numbers of samples of SF and urine were as follows: hyperparathyroidism 9 SF and 14 urine, hemochromatosis 6 SF (sufficient fluid from 2 knees of 1 patient) and 5 urine, hypomagnesemia 2 SF and 2 urine, hypothyroidism 11 SF and 20 urine, and normal controls 50 SF and 20 urine (Table 1). A dry tap was obtained in approximately 1 in 5 attempted aspirations. SF volumes varied from a few drops to 3.2 ml, and no untoward complications were experienced. In the hypophosphatasia group, only urine samples (n = 5) were obtained: 3 had symptomatic knee arthritis, and insufficient SF was obtained from the other 2.

CPPD crystals were identified in some SF samples (with or without accompanying isolated radiographic evidence of chondrocalcinosis) in each disease group (Table 1). Normal subjects over age 50 required normal radiographic findings (no chondrocalcinosis) before inclusion. Of the 50 joint fluids obtained from this group, none contained CPPD crystals.

The detection limit of the PP_i assay was tested and confirmed to 100 pmoles of PP_i. Specificity, as previously reported for this method (42), was demon-

strated by significant loss of radioactivity on addition of yeast pyrophosphatase to the final incubation. Recovery of added PP_i to SF (in triplicate measurements) ranged from 91–105% when concentrations of added PP_i ranged from 0.5–8 μmoles. During the period of these experiments, the coefficient of variation for the assay was 9% (same-day estimations on 10 aliquots of the same SF), and the mean coefficient of variation (n = 10) between triplicate measurements was 8%.

In normal control knees, the mean (±SEM) SF PP_i level was 10 ± 0.5 μM (Table 1). No association was observed between SF PP_i levels and age ($r = -0.04$). Elevated SF levels were seen in hypomagnesemia patients (27 ± 0.1 μM), hemochromatosis patients (23 ± 5 μM), and in patients with hyperparathyroidism (19 ± 3 μM) ($P < 0.05$ for all 3 versus controls), whereas reduced levels occurred in those with hypothyroidism (4.2 ± 2.3 μM) ($P < 0.05$ versus controls). Within each disease group, SF PP_i levels in samples containing CPPD crystals were no different from those of samples without crystals.

The mean (±SEM) urinary PP_i/creatinine in normal controls was 2.4 ± 0.4 μmoles/mmole. Elevation of urinary PP_i/creatinine was seen only in those with hypophosphatasia (5 ± 1 μmoles/mole) ($P < 0.05$ versus controls). Normal levels were found in those with hyperparathyroidism, hemochromatosis, and hypothyroidism ($P > 0.05$ versus controls), while low levels were seen in the 2 patients with hypomagnesemia (Table 1).

DISCUSSION

The radiometric assay system employed for PP_i estimation is both sensitive and specific (42,43) and contains internal and external controls for potential confounding variables such as hydrolysis of PP_i during the incubation phase of analysis. Collection, storage, and treatment of all urine and SF samples was identical, and the differences in values obtained between disease groups and normal subjects is therefore likely to reflect true in vivo differences in PP_i concentrations.

In respect to urinary PP_i findings, hypophosphatasia was the only disease to associate with elevated levels (corrected to creatinine), an observation first reported by Russell (37). The finding of normal urinary PP_i levels in hyperparathyroidism similarly concurs with previous studies by Russell and Hodgkinson (40) and Lewis et al (48), but contrasts with the elevated levels reported by Avioli et al (38,39). The reason for this discordance is uncertain, but may relate to differences in assay technique, correction of values to creatinine, or the severity of associated bone or joint disease (40). To our knowledge, urinary PP_i levels have not previously been estimated in hemochromatosis, hypomagnesemia, or hypothyroidism. The finding of non-elevated (normal) levels in hypothyroidism, however, is consistent with the reported elevation of urinary PP_i in the converse situation of hyperthyroidism (40). Although elevated blood and urinary PP_i levels are reported to occur in osteogenesis imperfecta (49), we did not include osteogenesis imperfecta or hyperthyroidism groups in this study, since these diseases have no suggested association with CPPD deposition, and elevation of PP_i levels in osteogenesis imperfecta has not been confirmed (40,41).

Urinary levels of PP_i are subject to influence by abnormalities throughout the skeleton, as well as by dietary and other confounding factors (32). Our study centered on the knee, and an extensive screen to characterize the presence of arthropathy or bone abnormality at other sites was not undertaken. Since CPPD crystal deposition occurs predominantly in the mid-zone of hyaline and fibrocartilage (1,2), the local concentration of PP_i in cartilage (rather than urine or plasma) is of greatest interest in respect to CPPD crystal formation. However, because of the impracticality of in vivo cartilage sampling, we estimated PP_i in the nearest accessible compartment, the synovial space. The study was confined to the knee since this is a common site for CPPD crystal deposition (1) and a joint that is readily aspirated in nonarthritic subjects. Elevated levels of SF PP_i were found in hyperparathyroidism, hemochromatosis, and hypomagnesemia groups, while low levels were found in the group with hypothyroidism. Appropriate SF samples in hypophosphatasia were not obtained. To our knowledge, this is the first report of SF PP_i estimations in metabolic diseases associated with CPPD crystal deposition.

Due to the rarity of some of the metabolic diseases investigated, exact age-matching between groups was not possible, and a wide age range of patients and normal subjects was therefore studied. However, the age differences between groups are not likely to be significant; knee SF PP_i levels in normal adults in this and another study (50) showed no age-associated change, and only mature adults were included for study. Since SF PP_i levels are increased in osteoarthritic, structurally damaged, knees (43–47), we were careful to include only asymptomatic, clinically normal, knees and radiographically assessed for

unsuspected structural abnormality in all patients with metabolic disease and in older normal controls, excluding those with positive findings. The definition of "normal," however, is problematic, and it remains possible that some metabolic disease patients and normal subjects had subclinical joint disease. The presence of isolated CPPD crystals or chondrocalcinosis in asymptomatic knees of some patients was the only abnormality detected in such joints, though this did not appear to influence the SF PP_i findings. Although other, more subtle, abnormalities cannot be excluded, it seems most likely that the differences in SF PP_i values demonstrated between groups in this study primarily reflect the direct or indirect influence of metabolic disease alone on the metabolism of inorganic pyrophosphate.

SF PP_i concentration was estimated at a single time point, with no attempt to determine the kinetics of production, breakdown, or clearance from the synovial space. Nevertheless, assuming a state of dynamic equilibrium between SF and cartilage (32,44), changes in SF PP_i are likely to reflect similar changes in cartilage, though the relationship need not be linear. If, as suggested, chondrocytes are a major source of extracellular PP_i within articular tissues (released during the breakdown of nucleoside triphosphates by the ectoenzyme NTP pyrophosphatase [33]), the concentration of PP_i in cartilage may indeed be higher than that in SF. The elevated SF levels in the present study are therefore likely to reflect similar, or even greater, changes in cartilage at the site of crystal formation.

This study provides direct evidence for alteration of PP_i levels in articular tissues by metabolic diseases (hyperparathyroidism, hemochromatosis, hypomagnesemia) that predispose to CPPD deposition and confirms the presence of increased urinary PP_i levels in another metabolic disease (hypophosphatasia). An increase in the ionic product ($PP_i \times Ca^{2+}$) may thus be one mechanism that predisposes to CPPD crystal deposition in such conditions, as has been suggested (32). Conversely, the finding of low SF and normal urinary levels of PP_i in hypothyroidism must further question, as do the findings of recent radiographic surveys (51,52), the putative association between this disorder and CPPD crystal deposition (30,31).

Although elevated PP_i levels have been demonstrated in this study, other factors which may play an equal or more important role in predisposition to crystal formation were not assessed. Investigation of tissue factors which promote or inhibit crystal nucleation and growth remains difficult, though involvement of such factors is likely to be crucial to CPPD crystal formation in sporadic (44) and certain familial (10, cases, as well as in metabolic diseases.

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FAMILIAL CHONDROCALCINOSIS DUE TO CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION IN ENGLISH FAMILIES

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SUMMARY

Familial predisposition to chondrocalcinosis (CC) due to calcium pyrophosphate dihydrate (CPPD) crystal desposition is described in five English kindreds. Two families were characterized by premature-onset polyarticular CC with little associated structural arthropathy. In one of these families, recurrent childhood fits were strongly associated with subsequent development of CC. Affected members of the other three families resembled sporadic disease in showing predominantly late-onset, oligoarticular CC with mild arthritis and destructive change in only one case. Knee synovial fluid levels of inorganic pyrophosphate (PPi) and nucleoside triphosphate pyrophosphatase (NTPP) did not differ from those of 59 sporadic cases of CC due to CPPD, although PPi and NTPP levels in both groups were higher than in normal knee synovial fluid ($P<0.0001$). Urinary PPi levels were not different from normal controls. Screening for other metabolic abnormality was negative in all cases.

This is the first report of familial CC in the UK, and the first to associate this condition with childhood fits. Absence of overt primary abnormality of PPi metabolism suggests that other factors relating to crystal nucleation/growth may be more relevant to predisposition in these cases.

Key words: Pseudogout, Fits.

Deposition of calcium pyrophosphate dihydrate (CPPD) crystals in articular cartilage is a common predominantly age-related phenomenon [1, 2]. CPPD crystals are the usual cause of radiographically detectable chondrocalcinosis (CC) and their presence may be associated with characteristic acute and chronic arthritis ('CPPD crystal deposition disease', 'pyrophosphate arthropathy' [1–3]). The precise physicochemical factors that result in CPPD deposition are unknown although recognized associations include ageing [1, 2], preceding joint insult [4], certain metabolic diseases [5], and familial predisposition [6–30]. Most cases of CPPD deposition are 'sporadic', having no overt metabolic or familial predisposition.

Familial or hereditary CC has been reported from various countries and ethnic groups including Czechoslovakia [6–8], Chile [9–11], Holland [12–14], France [15, 16], Canada [17], Germany [18], Sweden [19, 20], United States [21–23], Spain [24–28], Japan [29] and Israel [30]. Two different clinical phenotypes have been emphasized, the first characterized by early onset, polyarticular involvement, with variable severity of arthropathy and the second by late-onset oligoarticular CC with arthritis resembling sporadic disease [24–27]. The inheritance pattern also varies, although autosomal dominance is suggested in most. Only two studies have investigated the possibility of an inherited abnormality of inorganic pyrophosphate (PPi) metabolism in familial disease [31, 32]. Both reported an increase of intracellular concentration of PPi in fibroblasts (as well as lymphoblasts in one series) cultured from affected family members of one French [31] and three American [32] kindreds. This supports a generalized metabolic defect expressed phenotypically only in cartilage.

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We report the clinical and radiographic features of five affected families, the first to be described in the UK. Abnormalities of extracellular PPi metabolism were sought by estimation of synovial fluid and urinary PPi levels with comparison to values obtained from sporadic CC and normal controls. Nucleoside triphosphate pyrophosphatase (NTPP) is an ectoenzyme involved in salvage of extracellular nucleoside triphosphates by conversion to monophosphates, which are readily reabsorbed, and PPi [33]. The activity of NTPP in synovial fluid was also estimated because of its importance in the production of extracellular PP [33–35].

PATIENTS AND METHODS

The five unrelated families were detected following presentation of index cases to Buxton (Family 1), Telford (Family 2) or Nottingham (Families 3, 4, 5).

Available first degree relatives were questioned and examined in respect of locomotor and medical problems. Radiographs of knees and hands were undertaken in asymptomatic adult relatives to screen for CC. In chondrocalcinotic, symptomatic or clinically arthropathic subjects the radiographic screen was broadened to include additionally pelvis, shoulders and cervical, thoracic and lumbar spine, plus other clinically involved sites. Routine metabolic screening of affected subjects included full blood count, alkaline phosphatase, calcium, magnesium, ferritin, uric acid and thyroid function.

Knee synovial fluid (SF) was aspirated on at least one occasion from all affected subjects. Crystal identification was by characteristic morphology and birefringence using polarized light microscopy of fresh unspun SF. SF samples were centrifuged at 2500 *g* for

15 min to remove cells and crystals: supernatants were stored at −20°C and assayed within 2 months for inorganic PPi levels (μM) and activity of NTPP (μM of PPi/30 min/mg total protein) using a modification [36] of the radiometric assay developed by McGuire *et al.* [37]. Second void fasting morning urines were similarly frozen and assayed for PPi with correction for creatinine (μM PPi/mM creatinine).

Controls for SF PPi and NTPP findings were:

(1) knee SF samples from 59 patients (29F, 30M) with symptomatic 'sporadic' pyrophosphate arthropathy (SF CPPD crystals ± radiographic CC), having no evidence of predisposing metabolic disease and no suggestive family history (pyrophosphate arthropathy and CC involved no more than four joint sites in any patient, and were confined to one or both knees in 37);
(2) knee SF from 50 normal volunteers (29F, 21M [26]) with no symptoms or signs of knee disease (knee radiographs were obtained in those aged >50 to exclude occult CC); and
(3) urines from 12 of the normal volunteers.

Statistical comparison between the continuously variable data was by Wilcoxon rank sum test.

RESULTS

Of 33 invited relatives, 29 agreed to participate and in addition, joint radiographs were available for two deceased relatives with clinical documentation in one. The five kindreds are outlined in Figs 1 and 2. Family 1 originated from Belfast, Northern Ireland, where ancestors dated back at least as far as the early nineteenth century. Families 2–5 had apparently dwelt in the Midlands for at least four preceding generations, although prior origins were not traced. Family names and anthropological characteristics suggested nothing other than British ancestry. There was no evidence of consanguinity in any family.

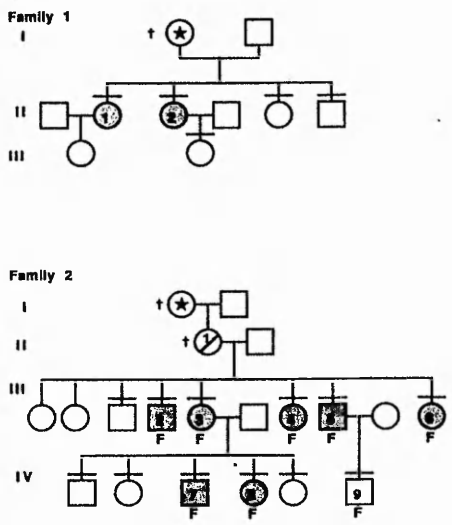

FIG. 1.—Pedigrees of the two families with premature onset polyarticular chondrocalcinosis. Numbers I–IV represent generations (circles = women, squares = men). Examined subjects are indicated by horizontal bars, deceased subjects by an adjacent cross. Shading represents chondrocalcinosis + synovial fluid confirmation of CPPD; a diagonal bar alone represents chondrocalcinosis without synovial fluid crystal confirmation. Stars signify deceased subjects with unconfirmed historical evidence of similar arthropathy. In Family 2 'F' signifies occurrence of childhood fits.

In the five families, 15 subjects had radiographic CC and confirmed SF CPPD crystals ('definite' pyrophosphate arthropathy); three (two deceased) had radiographic CC without available SF confirmation; two had SF CPPD crystals without CC; and in four deceased subjects there was a clear history (but no investigative evidence) of joint disease similar to that of affected first degree relatives.

Clinical and radiographic features of arthropathy

The clinical features of living subjects with arthropathy are summarized in Table I. Radiographic findings of living and deceased affected subjects are shown in Table II.

Families 1 and 2 both showed early onset under age 50 with symptoms beginning and predominating in knees but later coming to involve multiple joint sites. Recurrent acute attacks ('pseudogout') were the usual initial presentation at each site, later continuing superimposed upon a background of chronic or intermittent arthralgia. Chronic inflammatory arthritis with persistent effusions or deformity did not develop, and functional outcome in general was good. In addition to sites frequently involved by pyrophosphate arthropathy (knees, wrists, shoulders) less common sites (elbows, hips, ankles) were also well represented. The cervical spine was particularly troublesome in 7/10 affected members, three describing symptoms of acute pseudogout at this site (two with meningism). Radiographs showed widespread, florid CC affecting fibro- and hyaline cartilage of common and less commonly affected joints (Figs 3, 4), often with accompanying capsular and synovial calcification. In the spine, both annulus and nucleus calcification was observed (Fig. 5). Structural changes of arthropathy (osteophyte,

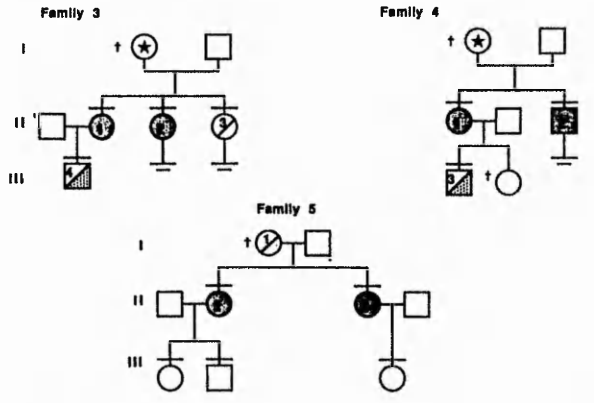

FIG. 2.—Pedigrees of Families 3, 4 and 5 (double horizontal bars indicate married with no children; a diagonal bar with shading indicates synovial fluid CPPD crystal identification with no chondrocalcinosis; otherwise same key as in Fig. 1).

TABLE I
PRINCIPAL CLINICAL FEATURES OF ARTHRITIS IN THE 5 FAMILIES

Family, member		Sex	Symptom onset	Acute attacks	Chronic symptoms	Principal affected joints				
						Knees	Wrists	Shoulders	Neck	Other
1	1	F	5th	+	+	+	+	+	+	Elbows
	2	F	5th	+	+	+	+	+	+	—
2	1	F	4th	+	+	+	+	+	+	Elbows, hip
	2	M	4th	+	+	+	+	+	−	—
	3	F	4th	+	+	+	+	+	+	Elbows
	4	F	3rd	+	+	+	−	+	+	Ankles
	5	M	3rd	+	−	+	−	−	−	Hip
	6	F	3rd	+	−	+	−	+	+	Elbows
	7	M	3rd	+	−	+	−	+	−	—
	8	F	3rd	+	−	+	+	+	+	—
3	1	F	7th	+	+	+	−	−	−	—
	2	F	6th	+	+	+	−	−	−	—
	3	F	—	−	−	−	−	−	−	Asymptomatic (age 70)
	4	M	4th	+	+	+	−	−	−	—
4	1	F	7th	−	+	+	−	−	−	Interphalangeal joints
	2	M	6th	+	+	+	−	−	−	—
	3	M	6th	+	−	+	−	−	−	—
5	2	F	6th	+	+	+	−	−	−	—
	3	F	5th	−	+	+	−	−	−	Hip

sclerosis, cyst, joint space narrowing) were predominently absent or only modest (Figs 3, 4).

Families 3, 4 and 5 were characterized by later onset of symptoms (usually over age 50) largely confined to knees. Acute attacks were common, but chronic inflammatory knee arthropathy was the major problem in 7/9 subjects. Radiographs showed CC largely confined to knees, wrists or symphysis pubis, with associated capsular or synovial calcification in two; polyarticular CC occurred in only two cases (one with spinal involvement). Variable accompanying structural changes of osteoarthritis were observed, mainly in knees, but destructive gonarthrosis with marked cartilage and bone attrition was seen in only one (subject 5, 2). Coexisting joint disease or insult was identified in two cases: patient 4,1 had apparent coexisting nodal generalized osteoarthritis (OA) with typical Heberden's and Bouchard's nodes, and patient 4,3 had undergone unilateral meniscectomy at age 22 and subsequently developed isolated 'post-meniscectomy OA' with SF CPPD crystals but no detectable CC.

Routine screening for predisposing metabolic disease was negative in all families.

Association with fits in Family 2

Multiple childhood fits between ages 6 months and 6 years, occasionally but not inevitably associated with febrile episodes, had occurred in all members of

TABLE II
DISTRIBUTION OF CHONDROCALCINOSIS IN THE 5 FAMILIES

Family, member		Chondrocalcinosis									Associated arthropathy
		Knee	Wrist	Symphysis	Shoulder	Spine	Hip	Elbow	Ankle	Other	
1	1	+	+	+	+	+	+	+	+	MCPJ,CMCJ	Mild
	2	+	+	+	+	+	+	+	+	MCPJ,CMCJ	Mild
2	1	+	+	+	+	+	+	+	+	ACJ,CMCJ	Mild
	2	+	+	+	+	+	+	−	−	MCPJ,SIJ	Mild
	3	+	+	+	+	+	+	+	−	MCPJ,CMCJ	Mild
	4	+	+	+	+	+	+	+	−	CMCJ	Mild
	5	+	+	+	−	−	+	−	−	CMCJ	Mild
	6	+	+	+	−	+	+	−	−	—	—
	7	+	−	+	+	−	+	−	−	—	—
	8	+	+	+	−	−	−	−	−	—	—
3	1	+	−	−	−	−	−	−	−	—	Moderate
	2	+	+	+	+	+	+	−	−	—	Mild
	3	+	−	+	−	−	−	−	−	—	—
4	1	+	−	+	−	−	−	−	−	—	Moderate
	2	+	−	+	−	−	+	+	−	—	Moderate
5	1	+	+	+	−	−	−	−	−	—	Moderate
	2	+	+	+	−	−	−	−	−	—	Severe
	3	+	+	+	−	−	−	−	−	—	Mild

MCPJ, metacarpophalangeal joint; CMCJ, carpometacarpal joint; ACJ, acromioclavicular joint; SIJ, sacroiliac joint.

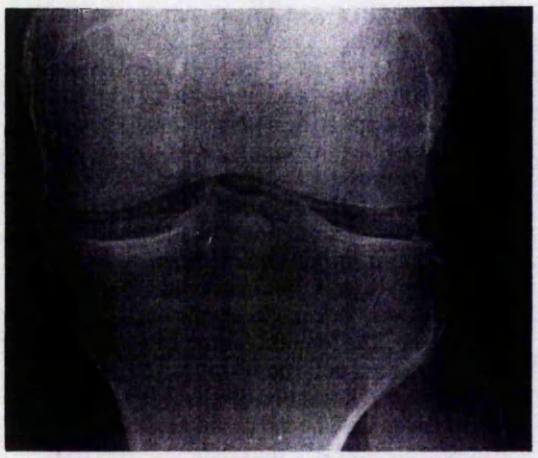

FIG. 3.—Weight bearing knee radiograph of 58-year-old male (patient 2, 2) showing marked chondrocalcinosis of both tibiofemoral compartments (hyaline and fibrocartilage) and the superior tibiofibular joint (a rare site for chondrocalcinosis). Note the absence of structural arthropathy.

Family 2 who subsequently developed CC. None was left with obvious mental handicap. Comparison with adult non-CC relatives (Fig. 1) showed the association between childhood fits and CC to be 100% (subject 9 is only 4 years old).

PPi and NTPP estimations

No differences in PPi or NTPP levels were observed in SF from clinically uninflamed knees of familial and sporadic CPPD patients (Table III), although PPi and NTPP levels in both groups were higher than in normal knee SF ($P<0.0001$). There was no difference between familial CPPD patients and normal controls for urine levels of PPi corrected for creatinine (median, range: 1.24, 0.5–3.85, and 2.54, 0.5–3.81 μM PPi/mM creatinine respectively).

DISCUSSION

This is the first report of familial CC and CPPD crystal deposition in the UK. As with previous reports [6–30] two different phenotypes were observed. Families 1 and 2 were typical of the florid, easily recognized form with premature-onset acute attacks, polyarticular involvement, and exuberant CC with little associated structural arthropathy—a pattern that should always lead to a search for familial or metabolic predisposition [3]. The other three families, however, were more typical of the late-onset, pauciarticular form that is clinically and radiologically indistinguishable from sporadic pyrophosphate arthropathy [24–28]. Recognition of familial predisposition in the latter is particularly hampered by late onset of disease expression and frequent geographical dispersal of families, and it may be that familial factors in apparent 'sporadic' disease are more common than is generally realized [24]. Prior meniscectomy is known to predispose to CPPD deposition [4], and it is tempting to speculate that additional predisposing familial factors in patient 4,3 combined to result in isolated CPPD crystal deposition at a young age (32 years). Although numbers are small and a systematic radiographic survey of all adult family members was not possible, the inheritance patterns (particularly evident in the larger Family 2) are consistent with autosomal dominant transmission.

Families 1 and 2 demonstrated predominant acute attacks and arthralgia, rather than chronic deforming arthropathy, and are thus similar to the more benign forms of polyarticular CC described in Holland [12–14], Sweden [19, 20], Canada [17] and the USA [21–23]. Family 1 appeared to originate in Ireland; although precise geneologies were not traced there were no known recent connections with mainland Europe or with Jewish ancestry [23, 30]. Vertebral involvement in familial CC has previously been

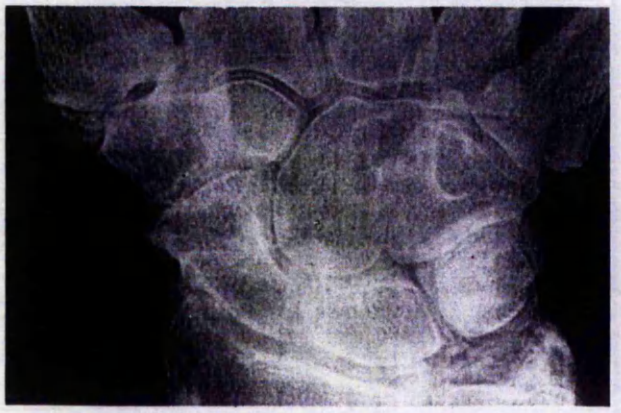

FIG. 4.—Wrist radiograph of 53-year-old female (patient 2,4) showing marked isolated chondrocalcinosis of triangular ligament and radiocarpal, intercarpal and carpometacarpal joints. Dense capsular/synovial calcification of the first carpometacarpal joint is also present.

FIG. 5.—Lateral cervical spine radiograph of patient 1,1 showing marked intervertebral calcification in the absence of gross structural change.

TABLE III
SYNOVIAL FLUID PYROPHOSPHATE (PPi) AND NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHATASE (NTPP) FINDINGS IN FAMILIAL AND SPORADIC CPPD CRYSTAL DEPOSITION

	Familial CPPD	Sporadic CPPD	Normal
(n =)	(16)	(59)	(50)
PPi: median, interquartile range (μm)	23 13.5–29	16 10.5–28	9.5 8–12
NTPP: median, interquartile range (μm PPi/30 min/mg protein)	2.6 1–3.9	2.2 1.4–3.3	1 0.4–2

emphasized [6–8, 23], and in both polyarticular families cervical spine symptoms were prominant. Axial acute attacks associated with meningism, similar to those experienced by three of our subjects, have previously been described in relation to non-familial CPPD deposition in cervical ligamentum flavum [38]. Unlike some other polyarticular CC families there was no tendency to vertebral or peripheral ankylosis [23, 39].

Association between benign childhood fits and familial CC has not previously been reported. Although CPPD crystal deposition has been described in dura mater [40] the young age involved, temporal separation between fitting and development of CC, and the self-limiting nature of the fits strongly argue against a direct association with CPPD crystals. A more plausible explanation is either genetic linkage between closely situated alleles independently determining fits and CC, or a genetically determined metabolic abnormality that results in both abnormalities. To our knowledge congenital hypomagnesaemia due to isolated or multiple renal tubular defects is the only recognized metabolic abnormality that predisposes both to premature CC and fits [41, 42]. Serum magnesium estimations, however, were normal in all affected subjects, including a 4-year-old with current fits (subject 2,9). Nevertheless, the possibility of a more subtle, undetected, metabolic defect remains.

The urine and SF PPi estimations support neither a generalized nor compartmentalized primary abnormality of PPi metabolism in these families. Assay insensitivity is an unlikely explanation for the negative urine findings since this system [36, 37] is able to detect increased urinary PPi in hypophosphatasia [43], a metabolic disease predisposing to CPPD deposition. Elevated PPi is previously reported in SF but not urine or plasma of patients with sporadic pyrophosphate arthropathy [1, 33, 35, 36], the level being influenced by the inflammatory state of the joint [36]. Our finding of similar SF results in familial and sporadic forms for both PPi and NTPP, an enzyme important in extracellular production of PPi [33–35], lends little support for even a compartmentalized primary abnormality of PPi in familial cases. It is of course possible that some unsuspected familial cases were included in 'sporadic' controls. Even so, there was no obvious difference in SF PPi or NTPP between patients with florid, dense polyarticular CPPD and those with modest, limited crystal deposition.

Lust *et al.* [31] reported increased intracellular PPi levels in lymphocytes and skin fibroblasts of French familial cases, supporting a generalized abnormality of PPi metabolism in that family. Ryan *et al.* [32] found elevated intracellular PPi but normal NTPP activity in skin fibroblasts of three American kindreds: similar elevation of PPi, accompanied by increased activity of NTPP, was found in patients with sporadic pyrophosphate arthropathy. CPPD crystal nucleation and growth, however, occur predominantly in perichondrocyte matrix [33, 34], and since PPi appears unable to transport across cell membranes [33], extracellular rather than intracellular concentration is likely to be of more relevance. Cell death could secondarily result in elevated extracellular PPi, although histological study reveals predominantly hypertrophic rather than dead chondrocytes at sites of familial and sporadic CPPD deposition [44]. Cartilage matrix factors that influence crystal nucleation and growth are a likely alternative explanation for differences in crystal deposition between individuals [2, 3, 44, 45]. Such factors, which may include proteoglycan or lipoprotein determinants [44, 45], could come under genetic influence, directly or indirectly, and be the inherited factor in families such as ours. Nevertheless, multiple factors affect crystal formation and the marked heterogeneity of familial CC suggests that different mechanisms of predisposition may apply in different families.

ACKNOWLEDGEMENTS

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Synovial fluid pyrophosphate and nucleoside triphosphate pyrophosphatase: comparison between normal and diseased and between inflamed and non-inflamed joints

Martin Pattrick, Edith Hamilton, June Hornby, Michael Doherty

Abstract

Deposition of intra-articular calcium pyrophosphate is associated with both aging and arthropathy; increased concentrations of free pyrophosphate (PPi) may contribute to such deposition. Free pyrophosphate and nucleoside triphosphate pyrophosphatase (NTPase) were estimated in synovial fluids from 50 subjects with normal knees and from 44 patients with rheumatoid arthritis, 61 with pyrophosphate arthropathy, and 59 with osteoarthritis. For arthropathic knees clinically assessed inflammation was classified as active or inactive using a summated score of six clinical features.

The order of PPi (μmol/l) and NTPase (μmol PPi/30 min/mg protein) was pyrophosphate arthropathy> osteoarthritis> rheumatoid arthritis (median PPi, NTPase respectively: for pyrophosphate arthropathy 15·9, 0·45; for osteoarthritis 9·3, 0·25; for rheumatoid arthritis 4·4, 0·18), with significant differences between all groups. In pyrophosphate arthropathy both PPi (μmol/l) and NTPase (μmol PPi/30 min/mg protein) were higher than normal (15·9, 0·45 *v* 8·6, 0·2 respectively), but findings in osteoarthritis did not differ from normal. The inflammatory state of the knee had a distinct but variable effect on synovial fluid findings in rheumatoid arthritis and pyrophosphate arthropathy, but not in osteoarthritis. There was no correlation of either PPi or NTPase with age, or between PPi and NTPase in any group.

This study provides in vivo data for synovial fluid PPi and NTPase. It suggests that factors other than PPi need to be considered in a study of crystal associated arthropathy. Clinical inflammation, as well as diagnosis, is important in synovial fluid studies.

Although intra-articular deposition of crystalline calcium pyrophosphate dihydrate (CPPD) most commonly occurs as an identical, age related phenomenon,[1] such deposition has been associated with 'pyrophosphate arthropathy', a subset of osteoarthritis characterised by typical clinical and radiographic features.[2 3] The relation between crystals, inflammation, and arthropathy, however, remains uncertain.[1–3] The two principal hypotheses suggest that CPPD crystals either are (*a*) primary pathogenic particles or (*b*) are formed as a secondary event to joint insult, reflecting processes that accompany the articular response that is recognised as 'osteoarthritis'.[1]

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Many factors may affect crystal deposition and possibly, intra-articular inorganic pyrophosphate (PPi) concentration is important. Previous studies have estimated PPi in various arthropathies and reported raised concentrations in osteoarthritis synovial fluid. A possible source of excess PPi is through hydrolysis of nucleoside triphosphates spilled by metabolically active, dividing or damaged cells. This might be facilitated by activity of the chondrocyte ectoenzyme nucleoside triphosphate pyrophosphatase (NTPase), which is found 'free' in synovial fluid from osteoarthritic joints.[4–16] Estimation of PPi and NTPase in biological systems is technically difficult, however[16–21]; various methods have been used, with conflicting results.[22–27] Furthermore, sample numbers in most studies have been limited, and there is a paucity of data for normal joints.[5]

We used a modification of the sensitive and specific assay for PPi developed by McGuire *et al*[6] to estimate PPi and NTPase in knee synovial fluid from well defined patient groups and from normal controls. For abnormal knees the degree of clinical inflammation at the time of aspiration was assessed by a summated score using six variables[28]; comparison between inflamed and non-inflamed knees, as well as between diagnostic categories, was therefore made.

Subjects and methods

Approval for aspiration of normal knees (by means of the medial infrapatellar approach) was obtained from the local ethical committee.

NORMAL SUBJECTS AND PATIENTS

Normal volunteers (29 female, 21 male; median age 44, interquartile range 26–63 years) gave informed consent and had no symptoms or clinical signs of knee disease; radiographs were obtained in those aged >50 years to exclude occult osteoarthritis or other abnormality. All patients with rheumatoid arthritis fulfilled American Rheumatism Association criteria for classic or definite disease (29 female, 15 male; median age 63, interquartile range 54–69 years). Patients with knee osteoarthritis (29 female, 30 male; median age 70, interquartile range 63–75 years) had symptomatic gonarthrosis with weightbearing knee radiographs showing cartilage loss plus subchondral sclerosis, osteophyte, or both; none had radiographic chondrocalcinosis, synovial fluid CPPD crystals, or evidence of other primary joint disease. Patients

with chronic pyrophosphate arthropathy (40 female, 21 male; median age 77, interquartile range 72–83 years) had persistent (>3 months) symptomatic gonarthrosis with synovial fluid CPPD crystals and radiographic features of osteoarthritis (with or without chondrocalcinosis).

ASSESSMENT OF CLINICAL INFLAMMATION

For patients with rheumatoid arthritis, osteoarthritis, and pyrophosphate arthropathy each knee was designated as either 'active' or 'inactive' according to clinically assessed inflammation, using a summated score (0–6) of six features: duration of early morning and inactivity stiffness, warmth, joint line tenderness, effusion, and synovial thickening.[28] Scores of 4–6 were regarded as active, 0–2 as inactive; knees with intermediate scores were not included in this study.

SAMPLE HANDLING

All fluids were taken into sterile plastic containers and processed as soon as possible (within two hours). Crystal identification was by characteristic morphology and birefringence using compensated polarised light microscopy of unspun synovial fluid. Samples were centrifuged at 2500 *g* for 15 minutes to remove cells and crystals; supernatants were stored at −20°C and assayed within three months.

ASSAY OF INORGANIC PYROPHOSPHATE

Inorganic pyrophosphate was estimated by a modification of the radiometric assay developed by McGuire *et al.*[16] Briefly, the sample PPi was allowed to react with added tritiated uridine diphosphogluconate, and the product, glucose-1-phosphate, was converted to a labelled, stable, recoverable product—tritiated 6-phosphogluconate (each sample assayed in duplicate[29–32]).

The PPi assay mixture contained 57 mM TRIS acetate; 5·2 mM magnesium acetate; 4 μM nicotinamide-adenine dinucleotide phosphate; 18·6 μM glucose-1,6-diphosphate; 7·5 μM uridine diphosphogluconate; 0·4 U/l glucose-6-phosphate dehydrogenase; 0·2 U/l phosphoglucomutase; 0·136 U/l uridine diphosphoglucose pyrophosphorylase; and tritiated uridine diphosphogluconate (specific activity 110–370 GBq/mmol). A 100 μl aliquot of this mixture was incubated with 40 μl of either sample or standard PPi (0·05–0·8 μmol/l).

The incubation reaction was performed in triplicate for 75 minutes at 37°C, then terminated by cooling on ice. A 250 μl aliquot of activated charcoal solution (deionised water) was added to separate any unreacted substrate from labelled 6-phosphogluconate. After mixing, samples were centrifuged for 15 minutes at 2500 *g* at 4°C. Supernatant (200 μl) was counted for radioactivity in scintillation fluid.

ASSAY OF NTPase ACTIVITY

NTPase activity is expressed as μmol PPi (per mg total protein) generated over 30 minutes in the presence of added ATP.

Aliquots (200 μl) of synovial fluid were incubated at 37°C in the presence of 120 μM ATP and tracer ^{32}P PPI to determine any breakdown of PPi during subsequent incubation and extraction procedures.

The incubation mixture contained 5·4 mM KCl; 0·8 mM $MgSO_4$; 1·8 mM $CaCl_2$; 0·118 mM NaCl; 1 mM NaH_2PO_4; 5·56 mM glucose; and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid). The mixture was held for 30 minutes in a shaking water bath and then incubation was terminated by cooling on ice and by addition of 0·1 ml trichloroacetic acid (50% w/v). After centrifugation at 2500 *g* for 15 minutes at 4°C trichloroacetic acid was removed by the addition of one volume of tri-n-octylamine dissolved in three volumes of 1,1,2-trichlorofluoroethane. This mixture draws the trichloroacetic acid into the lower organic layer. The resulting 500 μl aqueous extract was neutralised by addition of 100 μl 0·1 M TRIS acetate/8 mM magnesium acetate.

To measure any hydrolysis of PPi to orthophosphate during incubation and extraction the following recovery procedure was carried out on each sample. Orthophosphate was removed by mixing 100 μl of each sample with an equal volume of ammonium molybdate (5% w/v) in 2·7 M HCl. The resulting phosphomolybdate complex was recovered by extraction into isobutanol/light petroleum (4:1 v/v). The remaining ^{32}P PPi was then counted in the aqueous phase in 0·1 M HCl. These reactions were performed in duplicate. Total synovial fluid protein was estimated by the Biuret method.

ANALYSIS

Differences between continuously variable data were tested by the Wilcoxon rank sum test, and correlation by Spearman's method.

Results

Table 1 shows the numbers of synovial fluids examined (one per subject) with subject characteristics. The three groups were divided into those with active and inactive joints. Median PPi and NTPase data are shown graphically in the figure. Table 2 provides an analysis of all the data. The principal findings were:

1 The order of PPi concentrations and NTPase activities was pyrophosphate arthropathy> osteoarthritis> rheumatoid arthritis, with highly significant differences between all groups ($p<0·001$).

2 In pyrophosphate arthropathy both PPi and NTPase were higher than in normal controls. In rheumatoid arthritis PPi concentrations were lower than in controls, though NTPase activities were the same. Results for osteoarthritis, however, were the same as those for normal controls.

3 The inflammatory state of the knee at the time of aspiration had little effect on synovial fluid findings in osteoarthritis. In pyrophosphate arthropathy, however, PPi concentrations were

Table 1 Median (interquartile range) of inorganic pyrophosphate and NTPase by disease category and clinical activity

	n	Age	M:F	Inorganic pyrophosphate (μmol/l)	NTPase (μmol PPi/30 min/mg protein)
Osteoarthritis					
Active	34			10·2 (4·7–17·2)	0·29 (0·14–0·39)
Inactive	25			6·8 (4·0–10·7)	0·24 (0·16–0·48)
Total	59	70 (63–75)	1:0·97	9·3 (5·7–15·8)	0·25 (0·14–0·42)
Pyrophosphate arthropathy					
Active	32			12·8 (9·3–15·9)	0·45 (0·24–0·64)
Inactive	29			25·0 (16·6–36·8)	0·41 (0·31–0·73)
Total	61	77 (72–83)	1:1·91	15·9 (10·5–28·8)	0·45 (0·29–0·68)
Rheumatoid arthritis					
Active	20			5·7 (4·5–8·9)	0·13 (0·07–0·19)
Inactive	24			1·7 (0·9–4·8)	0·27 (0·17–0·47)
Total	44	63 (54–69)	1:1·90	4·4 (1·5–8·3)	0·18 (0·10–0·29)
Normal	50	44 (26–63)	1:1·38	8·6 (6·4–10·7)	0·2 (0·08–0·42)

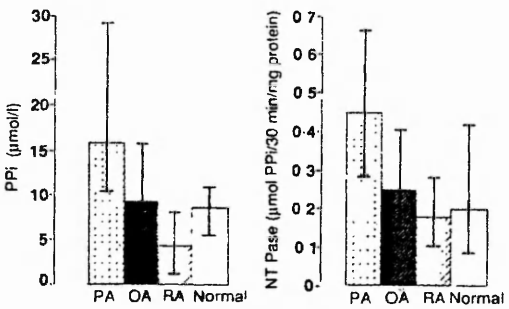
higher in inactive than in active knees, but there were no differences in NTPase. In rheumatoid arthritis, by contrast, PPi concentrations were higher in active joints, paradoxically accompanied by lower NTPase activity.

4 Neither PPi nor NTPase correlated with age in any group.

5 There was no correlation between the concentration of PPi and NTPase activity in any group.

Discussion

Several studies have examined PPi metabolism in patients with evidence of CPPD crystal deposition. Urine[7 32] and subsequently plasma[33 34] concentrations were reported to be the same as for normal controls. With development of more sensitive assays PPi concentration was estimated in synovial fluid and found to be greater than in matched plasma, the increase being reported to correlate with radiographic deterioration.[8] Smaller increases of synovial fluid PPi are also reported in osteoarthritis and in joints containing 'basic calcium phosphate' but not CPPD crystals[35]; the presence of consistently higher concentrations in synovial fluid than in matched plasma suggests local production within the joint. Synovial fluid NTPase activity has also been found to be increased when PPi is increased, suggesting that this enzyme is the major source of local PPi production.[1]



Median and interquartile range of inorganic pyrophosphate (PPi) and nucleoside triphosphate pyrophosphatase (NTPase) activity by diagnostic group.

Table 2 Significant findings for intergroup and intragroup comparisons

Between group comparisons		
PPi	PA>OA=Normal<RA	
	(p<0·001	(p<0·001)
NTPase	PA>OA, Normal, RA	(p<0·001)
	Normal<RA	(p<0·002)
Within group comparisons		
PPi	PA Inactive>PA Active	(p<0·001)
	RA Active >RA Inactive	(p<0·001)
NTPase	RA Inactive>RA Active	(p<0·002)

PPi=inorganic pyrophosphate; NTPase=nucleoside triphosphate pyrophosphatase. PA=pyrophosphate arthropathy; OA=osteoarthritis; RA=rheumatoid arthritis.

This study therefore accords with previous reports in showing (*a*) higher synovial fluid PPi concentrations and NTPase activities in pyrophosphate arthropathy than in osteoarthritis and (*b*) lower values in rheumatoid arthritis than in pyrophosphate arthropathy or osteoarthritis. For example, Rachow *et al*,[35] using the radiometric method of Cheung and Suhadolnik,[19] found significant differences in PPi concentrations in synovial fluid from various joints (knees, shoulders, hips) of 40 patients with osteoarthritis and 27 with pyrophosphate arthropathy (mean (SD) 14 (5) and 18 (8) μmol/l respectively); correspondingly higher NTPase activity was found in pyrophosphate arthropathy. Silcox and McCarty, using a differential colorimetric method, measured synovial fluid PPi in 35 patients with osteoarthritis, 29 with pyrophosphate arthropathy, and 12 with rheumatoid arthritis and found mean (range) concentrations of 9·2 (3–35), 9·8 (4–24), and 4·2 (2–8) μmol/l respectively[8]; the differences between rheumatoid arthritis and both osteoarthritis and pyrophosphate arthropathy were significant (Student's *t* test). Although these studies and ours agree about the differences between diagnostic categories, reported numerical values differ. This in part might reflect patient selection, differences in

PPi assay technique, and for NTPase estimations differences in correction to total (present study) rather than specific[26] proteins. Nevertheless, these differences are small and group values are within the same order of magnitude. Overall, such findings support the contention that local production of PPi relates to CPPD deposition, and that increased NTPase activity is a likely mechanism[2]; low values of PPi and NTPase in rheumatoid arthritis may also help to explain the negative association between rheumatoid arthritis and CPPD deposition.[36]

Certain observations, however, are not readily explained by NTPase regulated metabolism of PPi. In rheumatoid arthritis, for example, low concentrations of PPi occur in the presence of normal NTPase activity, and the lack of a direct correlation between PPi and NTPase in any group suggests that other factors may have a major effect on PPi concentrations. The importance of PPi metabolism in predisposition to sporadic CPPD crystal deposition must also be questioned.[37 38] Various factors may inhibit or promote crystal nucleation and growth,[37 39] and rates of crystal clearance and dissolution must be considered: the solubility product (calcium×PPi) may therefore not be the single most important determinant. The significance of factors apart from PPi is further emphasised by the discrepancy between the strong association between aging and CPPD deposition, and the apparent lack of age related change of PPi or NTPase in this study and in a larger cohort of normal subjects.[39] Temporal fluctuation in PPi concentrations is a further factor which has been poorly considered: differing increases of PPi influence not only CPPD but also apatite crystal formation,[40] and estimation at one timepoint may not be relevant to the crystals currently present. For this reason, cross-sectional studies relating single synovial fluid findings to 'radiographic damage'[8] are difficult to interpret, prospective studies being required to determine such relationships more precisely.

We recognise that there are inherent problems with all studies estimating concentrations in synovial fluid due to undetermined joint volumes and synovial mass, the unknown dynamics of production, breakdown and clearance, and the question of correction to other synovial fluid constituent concentrations. Furthermore, throughout such experiments it is generally assumed that synovial fluid concentrations reflect those in cartilage and we accept that this may not be valid.

In the study by Silcox and McCarty paradoxically lower concentrations of PPi in acute pseudogout than in chronic pyrophosphate arthropathy (n=18; mean 5·4 *v* 10·4 μmol/l) suggested an association between inflammation and PPi concentrations.[8] This was attributed to increased synovial flow and faster clearance of PPi. To date, however, few synovial fluid studies have characterised joints according to inflammatory state. There is no generally agreed method of clinically assessing knee inflammation, but the system we chose uses a summated score of six features and has been shown to correlate with complement activation in several arthritides.[28] Interestingly, we found the presence of such inflammation to have different associations in each condition: in rheumatoid arthritis it associated with increased PPi but lowered NTPase; in pyrophosphate arthropathy it associated with reduced PPi; and in osteoarthritis it had no discernible effect. Although such findings are difficult to explain, there are a number of ways in which inflammation might be expected to influence PPi metabolism (table 3) and the relative effects of these may vary in different conditions. The apparent lack of association with inflammation in osteoarthritis is of special interest: unlike rheumatoid arthritis, synovial fluid parameters, such as complement activation products[28] or PPi, were insensitive and did not reflect even florid clinical inflammation in osteoarthritis, suggesting that the mechanisms of inflammation in rheumatoid arthritis and osteoarthritis are different. Such discrepancies indicate that comparative synovial fluid studies should consider degree of inflammation as well as diagnostic categories.

Table 3 Factors influencing synovial fluid component concentration

1	Rate of PPi production
	NTPase, cytolysis, other nucleoside pyrophosphatases
2	Rate of PPi destruction
	Hydrolysis, enzymic (pyrophosphatase and lysosomal)
	Non-enyzmic (temperature and pH dependent)
3	Removal of PPi from synovial fluid
	Vascular, blood flow, lymphatic flow/stasis
	Leucocyte traffic
4	Effects on NTPase
	Proteolytic enzymes, pH, temperature

PPi = inorganic pyrophosphate; NTPase = nucleoside triphosphate pyrophosphatase.

Data on non-cadaveric, normal, human synovial fluids are understandably sparse. For PPi the results for only five normal joints have been published,[5] giving a mean value of 3·6 μmol/l; we are unaware of any data for NTPase. In this study, using a different assay, we found the mean PPi concentration from 50 normal knees to be 9·3 μmol/l; values for normal synovial fluid NTPase activity are also presented. Increases of synovial fluid PPi and NTPase in pyrophosphate arthropathy, compared with normal or osteoarthritis, may reflect increased biosynthetic activity within the joint rather than implying any specific alteration in PPi metabolism. Such an interpretation would accord with the 'hypertrophic response' (florid osteophytosis, cysts, remodelling) and possible good outcome[3] which have been associated with pyrophosphate arthropathy. Conversely, low synovial fluid concentrations of PPi in rheumatoid arthritis may reflect enzymic hydrolysis of PPi to orthophosphate, or an 'atrophic' articular response to insult.

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