

OLLI KAJANDER

Genetic and Environmental Factors
in Alcoholic Heart Muscle
Disease



ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their roman numerals. In addition, some unpublished data are also presented in the text and in the appendix.

- I. Kajander OA, Kupari M, Laippala P, Penttilä A, Savolainen V, Pajarinen J, Karhunen PJ (2001): Dose-dependent but non-linear effects of alcohol on the left and right ventricle. Revised version submitted to *Heart*.
- II. Kajander OA, Kupari M, Laippala P, Karhunen PJ (2001): Coronary artery disease modulates left ventricular remodeling due to heavy alcohol consumption. *Alcohol Clin Exp Res* 25:246-52.
- III. Kajander OA, Kunnas TA, Perola M, Lehtinen SK, Karhunen PJ, Jacobs HT (1999): Long-extension PCR to detect deleted mitochondrial DNA molecules is compromised by technical artefacts. *Biochem Biophys Res Comm* 254:507-14.
- IV. Kajander OA, Poulton J, Spelbrink JN, Rovio A, Karhunen PJ, Jacobs HT (1999): The dangers of extended PCR in the clinic. *Nat Med* 5:965-6.
- V. Kajander OA, Rovio AT, Majamaa K, Poulton J, Spelbrink JN, Holt IJ, Karhunen PJ, Jacobs HT (2000): Human mtDNA sublimons resemble rearranged mitochondrial genomes found in pathological states. *Hum Mol Genet* 9:2821-35.

ABBREVIATIONS

12S	12S ribosomal RNA
16S	16S ribosomal RNA
A6 and A8	A6 and A8 subunits of ATP synthase
ACE	angiotensin converting enzyme
ACM	alcoholic cardiomyopathy
AD	Alzheimer's disease
ad	autosomal dominant
ADH	alcohol dehydrogenase
AHMD	alcoholic heart muscle disease
ALDH	acetaldehyde dehydrogenase
AN(C)OVA	analysis of (co)variance
ANT	adenine dinucleotide translocator
ar	autosomal recessive
ATP	adenosine triphosphate
AT1R	angiotensin II type 1 receptor
AUDIT	Alcohol Use Disorders Identification Test
BSA	body surface area
CAD	coronary artery disease
CAGE	Cutting down, Annoyance at criticism, Guilty feelings, Eye-openers
CBR	common breakpoint region
CI	confidence interval
CMS	cytoplasmic male sterility
COI-III	COX subunits I-III
COX	cytochrome c oxidase
CYP(450)	Cytochrome P450
cyt b	cytochrome <i>b</i>
D-loop	displacement loop, non-coding region in mtDNA
DNA	deoxyribonucleic acid
FRDA	Friedreich's ataxia
HDL	high density lipoprotein
H-strand	heavy strand of mtDNA (according to buoyant density)
IBM	inclusion body myositis
IDC	idiopathic dilated cardiomyopathy
KSS	Kearns-Sayre syndrome
L-strand	light strand of mtDNA (according to buoyant density)

LV	left ventricle
LX-PCR	long extension PCR
MAST	Michigan Alcoholism Screening Test
MEOS	microsomal ethanol oxidation system
MI	myocardial infarction
MNGIE	mitochondrial neurogastrointestinal encephalopathy
MnSOD	manganese superoxide dismutase
MONICA	Monitoring of Trends and Determinants in Cardiovascular Disease (a WHO project)
mtDNA	mitochondrial DNA
mTERF	mitochondrial transcription termination factor
NAD ⁺	nicotinamide dinucleotide
NBT	nitrobluetetrazolium
ND1-6 and 4L	NADH dehydrogenase 1-6 and 4L
O _H	H-strand replication origin
O _L	L-strand replication origin
OXPPOS	oxidative phosphorylation
PCR	polymerase chain reaction
PEO	progressive external opthalmoplegia
P _H	H-strand promoter
P _L	L-strand promoter
POLG	DNA polymerase γ
RAA -system	renin-angiotensin-aldosterone system
RE	restriction endonuclease
RNA	ribonucleic acid
rRNA	ribosomal RNA
RV	right ventricle
SD	standard deviation
SEM	standard error of mean
SNP	single nucleotide polymorphism
TAS	termination associated sequence
Tfam	mitochondrial transcription factor A
tRNA	transfer RNA

ABSTRACT

Excessive alcohol use *per se* is known to be damaging to the myocardium, but the dose-dependence of different pathological alterations is not commonly agreed. It is commonly believed that heavy alcohol consumption may result in a form of dilated cardiomyopathy, called alcoholic cardiomyopathy (ACM). However, among alcoholics, the most common clinical cardiac findings have been left ventricular (LV) hypertrophy and mild systolic/diastolic dysfunction, rarely accompanied by ventricular dilatation resembling dilated cardiomyopathy. Although mostly asymptomatic, the myocardial abnormalities have been suspected to contribute to the greatly increased risk of arrhythmias and sudden cardiac death among heavy alcohol users.

To clarify the entity earlier termed alcoholic heart muscle disease (AHMD) or ACM, a large prospective series of middle-aged men, who died out-of-hospital, was studied via recourse to a medicolegal autopsy. Cardiac examinations for research purposes were added to a routine autopsy protocol to obtain detailed data on ventricular weights and dimensions and coronary artery anatomy. The calculated average daily alcohol dose was found independently to predict heart weight and right ventricular (RV) cavity size, but not LV size, among all men. In the subgroup of men free of coronary artery disease (CAD), LV cavity area showed with increasing alcohol doses a U-shaped response without change in weight (concentric remodeling), whereas RV cavity dilated with very heavy drinking. The LV concentric remodeling was modulated by CAD in a dose-dependent fashion, so that men with no or mild coronary narrowings presented a concentric change, while the opposite (eccentric remodeling) was seen in men with severe CAD. In all, the present results question the idea of progressive LV dilatation with increasing alcohol consumption among middle-aged men.

In heart, mtDNA rearrangements did not differ in any systematic manner between controls and cases with alcoholic cardiomyopathy diagnosed at autopsy. These low-abundance rearranged mtDNA molecules, found also in control myocardium, were termed sublimons, by analogy with plant mitochondria. Sublimons were in general most abundant in post-mitotic tissues, and certain sets of sublimons were specific to a given tissue. The structure of sublimons in heart was found closely to resemble pathological mtDNA rearrangements, introducing sublimon amplification as a potential new pathogenic mechanism in mitochondrial disease. In control heart, sublimons were detectable also by non-PCR methods, and like the pathological re-arrangements, sublimons were present in partially duplicated forms and also as deletion mono- and multimers. Rearrangement break-point hotspots were adjacent to known sites of protein binding in the 16S rRNA/tRNA(LeuUUR) gene boundary (by the mitochondrial transcription termination factor, mTERF), and near the end of the D-loop. This suggests the possibility of the involvement of protein-protein interactions in the generation of sublimons. In hearts of healthy individuals, sublimon abundance was found to vary over as much as three orders of magnitude, and in an autopsy series the most prevalent class of sublimons correlated independently with age and intensity of lifetime smoking. There was no correlation with any myocardial pathological finding, and thus it was proposed that sublimons, instead of being a causal factor in cardiac aging, may co-exist with wild-type mtDNA in an equilibrium which is regulated during aging by as yet unknown mechanisms.

INTRODUCTION

Acquired myocardial disease not caused by ischemic heart disease is most commonly associated with excessive alcohol use. Ethyl alcohol (ethanol) is the most commonly abused drug in Western society, its production, distribution and consumption being both socially and legislatively accepted. Alcohol abuse has unfavourable effects on various organs in the human body, including cardiovascular, central nervous, digestive and reproductive systems, and is the most important cause of increased mortality and morbidity among young males.

At the present, excessive alcohol use is believed to cause alcoholic cardiomyopathy (ACM), a poorly delineated entity in the subcategory of dilated cardiomyopathy, characterized by dilatation of all four chambers of the heart and leading finally to cardiac insufficiency. Subclinical cardiac abnormalities, often called alcoholic heart muscle disease (AHMD), are common findings among asymptomatic alcoholics without pre-existing ischemic or other heart disease. Although mostly asymptomatic, the incidence of atrial as well as life-threatening ventricular arrhythmias and sudden cardiac death is increased among alcoholics, most probably due to acute triggering factors superimposed on chronic structural, physiological and metabolic changes due to long-term abuse. Although it is known that clinical cardiac abnormalities do not simply increase with increasing duration and dosage of alcohol intake, *post mortem* data are almost completely lacking. The changes in cardiac anatomy and the occasional eventuation of AHMD in clinical heart failure may involve acquired or inherited predisposing factors, which are as yet poorly recognized, and assumed to be enriched in medico-legal autopsy material.

Abnormalities of mitochondrial structure in the myocardium have been a constant feature of AHMD, suggesting the involvement of factors impairing mitochondrial function in the pathogenesis. However, no such factors have been identified. Mutations in mitochondrial DNA (mtDNA) are the sole cause of some, mostly rare hereditary cardiomyopathies, and syndromic diseases caused by mtDNA point mutations or deletions also involve the heart. It has been claimed that somatic mtDNA mutations, mainly in the form of large-scale deletions, can accumulate in the heart and contribute to tissue aging and some forms of idiopathic cardiomyopathies. The involvement of myocardial mtDNA abnormalities in AHMD has not been studied earlier.

REVIEW OF THE LITERATURE

1. Cardiovascular morbidity and mortality associated with alcohol drinking

1.1. Alcohol and coronary heart disease

Risk of coronary artery disease (CAD) and myocardial infarction (MI) has been shown to be reduced among moderate drinkers in comparison to total abstainers (Moore and Pearson, 1986, Marmot and Brunner, 1991, Rimm et al., 1991, Maclure, 1993, Doll et al., 1994, Klatsky, 1994, Rimm et al., 1996, White, 1996, Rehm et al., 1997, Thun et al., 1997, Yuan et al., 1997, Mäkelä et al., 1997, Hart et al., 1999, Corrao et al., 2000). The main mechanism of protection from coronary heart disease has been considered to be elevation of high density lipoprotein (HDL) (Cauley et al., 1987, Suh et al., 1992, Hein et al., 1996, Rimm et al., 1999). A recent meta-analysis concluded that alcohol intake is causally linked to a lower risk of CAD via increased concentrations of HDL-cholesterol and apolipoprotein AI and decreased concentrations of fibrinogen (Rimm et al., 1999). Studies showing no association between HDL and alcohol consumption also exist (Seppä et al., 1992). Notably, the effect of alcohol is seen only as an elevation of the HDL3-fraction (Haskell et al., 1984, Sillanaukee et al., 1993), the role of which in coronary disease is not clear. Recent studies have, however, shown that both HDL2 and HDL3 levels associate with incidence of MI (Buring et al., 1992, Stampfer et al., 1993). In addition, some other actions of ethanol may be involved in the protection against MI via haemostatic factors. Ethanol reduces platelet aggregability (Haut and Cowan 1974, Mikhailidis et al., 1983, Jakubowski et al., 1988, Renaud et al., 1992), increases thromboxane-prostacyclin ratio (Landolfi et al., 1984) and the release of tissue plasminogen activator (Laug, 1983, Booyse et al., 1999, Miyamoto et al., 1999) and reduces fibrinogen (Meade et al., 1979) and platelet activator inhibitor 1 levels (Dimmitt et al., 1998).

1.2. Alcohol and hypertension

Alcohol consumption is associated with hypertension (Klatsky et al., 1977, Friedman et al., 1983, Klatsky et al., 1986, MacMahon, 1987, Beilin et al., 1996), which has been shown also in controlled prospective studies (Wittman et al., 1990, Ascherio et al., 1992). The effect of alcohol is independent of other risk factors, such as pre-existing hypertension, obesity or salt intake (Klatsky et al., 1977, 1986, Keil et al., 1991). Intervention studies have shown that abstinence lowers blood pressure both in subjects with (Potter and Beevers, 1984, Puddey et al., 1987, Ueshima et al., 1993) and without (Puddey et al., 1985a, Rakic et al., 1998) pre-existing elevated blood pressure. As a consequence, chronic alcohol use has been considered as a potential contributor to treatment-resistant hypertension (Maheswaran et al., 1992).

Blood pressure response for alcohol intake is biphasic (Kawano et al., 1992, Kojima et al., 1993, Abe et al., 1994) and casual measurements alone may not be sufficient to determine the whole effect (Maiorano et al., 1995, Aguilera et al., 1999). Several potential mechanisms have been raised to explain the alcohol-associated blood pressure elevation (MacMahon, 1987, Kojima et al., 1993). Altered Na⁺ metabolism (Coca, 1992, Kojima et al., 1993) and increased serum Ca²⁺ (Arkwright et al., 1984) may be involved. Acute ethanol administration increases circulating catecholamines (Eisenhofer et al., 1983) and also sympathetic activity (Grassi et al., 1989, van de Borne et al., 1997), which can experimentally be reduced by inhibiting central excitatory mechanisms by dexamethasone administration (Randin et al., 1995). Alcohol interferes also with the renin-angiotensin-aldosterone (RAA) -system by stimulating renin release (Ibsen et al., 1981, Puddey et al., 1985b).

1.3 Alcohol, arrhythmias and sudden cardiac death

Retrospective case-control studies (Rich et al., 1985) and controlled clinical studies (Koskinen et al., 1987, 1990) have shown excessive alcohol use to be more common among cases of idiopathic atrial fibrillation than controls. This applies also to other supraventricular tachyarrhythmias (Koskinen and Kupari, 1991).

Originally shown in small selected series, heavy alcohol use associated with ventricular tachyarrhythmias (Singer and Lundberg, 1972, Ettinger et al., 1978, Greenspon et al., 1979), with the potential to cause lethal ventricular fibrillation. In autopsy studies, increased risk of sudden death (Kramer et al., 1968, Randall, 1980, Penttilä et al., 1989) and sudden cardiac death (Särkioja and Hirvonen, 1984, Vihert et al., 1986) have been found to be associated with alcoholism. Epidemiological data have also shown that heavy consumers tend to die suddenly more often than moderate drinkers or teetotalers (Wannamethee and Shaper, 1992). A Finnish material collected in the 1970s showed sudden death incidence to increase with increasing alcohol use among subjects both with and without ischemic heart disease (Suhonen et al., 1987), whereas alcohol in another study, partly via hypertension, increased the risk only among subjects without ischemic heart disease (Wannamethee and Shaper, 1992).

2. Estimation of alcohol consumption

Reliability of the alcohol consumption data is crucial for the studies of alcohol-induced end-organ damage. Heavy alcohol consumption is known to affect several measurable biological variables (Salaspuro, 1986). Some more recently developed biological markers, such as carbohydrate-deficient transferrin exist (Sillanaukee et al., 1992), but their reliability in unselected alcoholic populations still remains an issue of debate (Allen et al., 1994, Salmela et al., 1994a), and only crude estimation of the alcohol intake is possible. Direct measurement of chronic alcohol consumption of human subjects is practically impossible, necessitating the use of other methods. The most accepted and applied approach

has been indirect measurement of alcohol consumption by questionnaires and interviews (Poikolainen 1994). Questionnaires designed specifically to screen alcohol abuse and/or measure the amount of alcohol consumed have been shown to be highly sensitive and specific instruments (Watson et al., 1984, Karhunen and Penttilä, 1990, Babor et al., 1992, Girela et al., 1994, McIntosh et al., 1994, Fiellin et al., 2000). Commonly used alcoholism screening tests have been CAGE (Cut, Annoyance, Guilty, Eye-opener) (Mayfield et al., 1974), MAST (Michigan Alcoholism Screening Test) (Selzer et al., 1975) and the more quantitative AUDIT (Alcohol Use Disorders Identification Test) (Fleming et al., 1991). These are applied directly on the study subject, but also a person close enough to the subject can give reliable information about the alcohol use. It has been shown that MAST scores obtained from patients or their family members are not significantly different (McAuley et al., 1978). Another alcoholism screening test incorporating 35 items showed a mean 76.5 % patient-spouse agreement and 98 % of the cases were correctly classified as alcoholic or non-alcoholic on the basis of spouse ratings (Davis and Morse, 1987). One other study showed good patient-spouse agreement in most items, excluding the age when the drinking had started and the consumption in last week before interview (Ray et al., 1984). Self-reports have been suspected to underestimate the true consumption (Poikolainen, 1985, Fuller et al., 1988) and the amount of alcoholic beverages consumed seems to be more validly estimated by others especially when the subject has lost control over drinking (Davis and Morse, 1987).

3. Metabolism of ethanol

As the ingested ethanol enters the stomach, it is subjected to oxidation by gastric alcohol dehydrogenase (ADH). This metabolism taking place in the gastric mucosa may exert some protection against the systemic effects of alcohol by preventing high peaks of blood ethanol concentration (Lieber et al., 1994). It probably also explains why the ingestion of dilute alcoholic drinks with meals raise the blood ethanol concentration more than the distilled ones (Roine et al., 1993, Lieber et al., 1994). The estimated fraction of gastric ethanol metabolism has varied from 0.5 to 50 %, and the importance of first-pass ethanol metabolism is highly controversial (Levitt and Levitt, 1994, Lieber et al., 1994). This has partly been explained by differences in study designs, while genetic variation (Baraona et al., 1991), sex (Frezza et al., 1990), some commonly used drugs like aspirin and cimetidine (Lieber, 1988, Roine et al., 1990, Gentry et al., 1999) and chronic gastritis (Salmela et al., 1994) also contribute. Recent studies suggest that the rate of gastric emptying might be an important factor (Oneta et al., 1998).

Ethanol is mainly metabolized in the liver, where two systems, ADH pathway and microsomal ethanol oxidation system (MEOS), act simultaneously on the entering ethanol molecules. In the cytosol of the hepatic cells ADH oxidizes ethanol to acetaldehyde, which is converted to acetate by acetaldehyde dehydrogenase (ALDH) in the mitochondria (Weiner et al., 1988). Both of these reactions consume one molecule of NAD^+ , so to oxidize one molecule of ethanol, two molecules of NAD^+ are reduced to NADH.

Cytochrome P450 (CYP 450) 2E1 is the principal component of MEOS, located in the endoplasmic reticulum of the hepatocytes. This system uses NADP^+ to oxidate ethanol to acetaldehyde and as a by-product, generates H_2O_2 and subsequently reactive oxygen species (Ekstrom et al., 1986, Tsutsumi et al., 1989). This enzyme system is inducible by chronic alcohol ingestion and the mechanism of induction seems to differ according to the level of achieved blood alcohol concentration (Badger et al., 1993, Ronis et al., 1993).

4. Biochemical and metabolic alterations associated with ethanol intake

Ethanol is a rich source of calories without other nutritional value. Metabolism of 1 g of pure alcohol gives ca. 30 kJ (7.1 kcal) of energy, which is more than carbohydrates but less than lipids of the same weight. After the postulated microbial oxidation of ethanol by the bacteria in the colon, there would be ca. 6.0-6.5 kcal/g to be used by the human body. The so called ethanol-induced thermogenesis leads to a loss of 20-25 % of the energy of the ingested ethanol, which is clearly more than the ca. 10 % loss of a mixed meal (e.g. Suter et al., 1994). However, it has been suggested that via decreased lipid oxidation habitual ethanol consumption is likely to favor weight gain in form of lipids (Suter et al., 1992). Among heavy consumers, ethanol is often used to replace other energy sources in the diet, and an imbalance of daily nutrients may result (Salaspuro, 1993). Ethanol is metabolized in the liver at the expense of other energy sources, i.e. carbohydrates, lipids and proteins. A major metabolic consequence of ethanol oxidation is an increase in NADH/NAD^+ redox potential in tissues. (Reviewed in Peters and Preedy, 1998).

Mitochondrial function of rodents has been impaired after chronic ethanol treatment (Bottenus et al., 1982, Wu et al., 1987). A study on baboon hepatic mitochondria showed clearly impaired cellular respiration by the time fatty liver had developed (Arai et al., 1984). Only minor changes in heart mitochondrial energy metabolites were reported in a rat study, abnormalities of the liver being predominant (Cunningham et al., 1989). In alcohol-fed animals, the function of mitochondrial respiratory complexes as a whole has been found to be only mildly impaired in the liver (Spach and Cunningham, 1987). In another study, mitochondrial oxidative phosphorylation (OXPHOS) enzyme complexes were decreased in the liver, but upregulated in the heart (Marin-Garcia et al., 1995). Regulation of mitochondrial ATPase (complex V) has been reported to be defective (Das and Harris, 1993), the and mitochondrial transcript levels altered (Kou and Cohen, 1998) in the heart following ethanol ingestion. The mtDNA encoded protein subunits of ATP synthase (A6 and A8) are decreased, but the parts encoded by nuclear DNA unaffected by ethanol in liver mitochondria (Coleman et al., 1994). Ethanol administration has been found to impair hepatic mitochondrial protein synthesis (Coleman and Cunningham, 1990, 1991), and mitochondrial, but not cytoplasmic ribosomal proteins are affected by chronic ethanol consumption (Cahill et al., 1996).

Human alcoholics without manifest cardiomyopathy had heart mitochondrial function comparable to that of controls in a recent study (Miro et al., 2000) and mitochondria from

muscle of chronic myopathic alcoholics did also not reveal any signs of dysfunction of oxidative phosphorylation (Cardellach et al., 1992).

A recent study suggested massive degradation of liver mtDNA following an alcoholic binge in mice (Mansouri et al., 1999), a result yet to be confirmed in other studies. Long-term (11-13 months) ethanol feeding has been reported to produce a 36-44 % decrease in the hepatic mtDNA content of rats, with increased 8-hydroxydeoxyguanosine (8-OHdG) and mtDNA single-strand break production (Cahill et al., 1999). However, in this study mtDNA content was not decreased after 1-5 months chronic ethanol ingestion (Cahill et al., 1999) and nuclear DNA/mtDNA ratio was not determined.

5. Alcohol, myocardium and alcoholic heart muscle disease

The first remarks on cardiac abnormalities in chronic heavy alcohol users were made over 100 years ago (Bollinger, 1884, Steell, 1893). For a long period of time alcoholic cardiomyopathy or alcoholic heart muscle disease (AHMD) was considered to be nutritional and related to thiamine deficiency (beri-beri). Thiamine has, however, been ineffective in treatment of most AHMD cases (Robin and Goldschlager, 1970) and further, other markers for nutritional status have not been significant determinators of cardiac abnormalities (Dancy et al., 1985, Estruch et al., 1993). According to the literature, a typical case of alcoholic cardiomyopathy resembles dilated cardiomyopathy, and heart failure symptoms dominate (Burch and Giles, 1971, DeMakis et al., 1974, Regan et al., 1977, Rubin, 1979, Moushmouth and Abi-Mansour, 1991). Only about 1-5 % of chronic alcoholics develop heart failure symptoms (Hartel et al., 1969), which however, due to the large number of heavy consumers, makes alcohol the most common cause of acquired non-ischemic cardiomyopathy in Western countries (Roberts et al., 1987).

5.1 Changes in structure and function of the heart in AHMD

The individual left ventricular (LV) dimensions are determined by several factors including body size, age, hypertension, diabetes and smoking (Henry et al., 1980, Hartz et al., 1984), which have been only partly or not at all adjusted for in most earlier studies of AHMD. Acutely ingested ethanol is known to cause depression of myocardial contractility (Guarnieri et al., 1990, Thomas et al., 1994). An independent association of chronic alcohol use with LV abnormalities has been shown (Dancy et al., 1985, Kupari et al., 1991). Fulminant cardiac failure due to alcohol has been infrequent, and alcohol consumption has more commonly been associated with subclinical cardiac abnormalities. In clinical studies, chronic alcoholics have had hypertrophy of the left ventricle (Askanas et al., 1980, Kino et al., 1981, Mathews et al., 1981, Kupari et al., 1991), and systolic (Spodick et al., 1972, Urbano-Marquez et al., 1989) and diastolic (Ahmed et al., 1980, Silberbauer et al., 1988, Kupari et al., 1990) dysfunction.

High prevalence of alcoholism among autopsied idiopathic dilated cardiomyopathy (IDC) cases has been shown (Roberts et al., 1987), but no causal relationship between alcohol and dilated cardiomyopathy has been established. Autopsy materials assessing AHMD have been small and selected (Brigden and Robinson, 1964, Alexander, 1966) or retrospective, including a highly selected population of alcoholics (Steinberg et al., 1981). One larger autopsy series has been published, reporting the pathological findings of only descriptively (Vikhert et al., 1986), and not accounting for quantity of alcohol consumption or cardiac dimensions. In general, the findings of the autopsy studies have been highly unspecific and their relation to the subject's alcohol use unclear and no quantitation has been performed.

5.2 Myocardial findings associated with AHMD

Mitochondrial alterations have been frequently reported in hearts of alcoholics (Hibbs et al., 1965, Tsiplenkova et al., 1986, Sudarikova et al., 1997). Animal models have shown changes in the ultrastructure of myocardial mitochondria after chronic exposure to ethanol, which include mitochondrial 'swelling', ruptured and disorganized cristae and in some cases collection of inclusions inside the mitochondrial matrix (Burch et al., 1971, Segel et al., 1975, Mattfeldt et al., 1980, Morvai and Ungvary, 1987, Cunningham et al., 1989). Myocardial histological and ultrastructural studies have shown various other unspecific changes, such as focal or diffuse fibrosis and lipid deposits (Ferrans et al., 1965, Hibbs et al., 1965, Alexander, 1966, Bulloch et al., 1972) and loss of contractile elements (Bulloch et al., 1972). Patients with AHMD have been shown to have milder histological abnormalities in comparison to idiopathic dilated cardiomyopathy (Teragaki et al., 1993), but most authors still think that AHMD is indistinguishable from other forms of myocardial disease (Olsen and Trotter, 1993).

Cardiac activities of α -hydroxybutyric dehydrogenase, creatine kinase, lactate dehydrogenase and malic dehydrogenase have been elevated in chronic heavy drinkers with dilated cardiomyopathy (Richardson et al., 1986). Acute ethanol administration in rats has been shown to increase circulating cardiac troponin-T, a marker of ischemia (Patel et al., 1995). Concerning AHMD, the causality of these alterations has not been evidenced, and they may represent an adaptive response to the biochemical changes in the body (Richardson et al., 1986). Acetaldehyde forms with various proteins adducts, which are immunogenic and antibodies against these adducts have been observed in one-third of alcoholic cardiomyopathy patients in one study (Harcombe et al., 1995).

5.3 Dose-dependency of AHMD

Earlier clinical studies of chronic alcoholics have suggested that the development of AHMD manifested as increased LV mass and systolic dysfunction requires at least 12 years of alcohol use at a high level (Kupari et al., 1991). One research center has reported a linear correlation of the total lifetime consumption of ethanol with LV mass and systolic dysfunction (Urbano-Marquez et al., 1989), but other comparable studies have failed to show

any clear correlation (Askanas et al., 1980, Kino et al., 1981, Kupari et al., 1991). Making the interpretation of dose-dependency even more difficult, most of the studies have not adjusted for the potential confounders of LV measurements (Askanas et al., 1980, Kino et al., 1981, Mathews et al., 1981, Silberbauer et al., 1988, Urbano-Marquez et al., 1989). Kupari et al. have re-interpreted the results by Urbano-Marquez et al. (1989), and stated that there might exist a threshold for the lifetime cumulative alcohol dose of ca. 20 kg/kg of body weight, after which systolic function clearly decreased (Kupari et al., 1991). In all, these studies have not shown any simple dose-response effect, the relationship of AHMD with alcohol use is probably non-linear (Kupari et al., 1991), and has still remained a matter of debate.

6. mtDNA and myocardial disease

The energy essential for cellular survival is provided by mitochondria via oxidative phosphorylation (OXPHOS). Fatty acids are used for most of the aerobic metabolism in the heart to generate ATP through the β -oxidation pathway, Krebs cycle and the respiratory chain. The main OXPHOS machinery locates to the mitochondrial inner membrane and consists of five enzyme complexes (I-V). Whilst the enzyme complex subunits of the respiratory chain are coded by both nuclear DNA and mtDNA, all of the numerous other mitochondrial enzymes and structural and transport proteins are derived from nuclear DNA.

Human mitochondrial DNA (mtDNA) is a 16568 bp circular genome coding for essential parts of the OXPHOS machinery, namely 13 proteins, which are essential components of the respiratory chain, 22 tRNAs and 2 rRNAs (Figure 1) (Anderson et al., 1981). Recently, a revised version of this so called ‘Cambridge’ reference sequence was published (Andrews et al., 1999). mtDNA is maternally inherited, contains only minimal non-coding areas (the D-loop), has a unique genetic code and has been estimated to be present at thousands of identical copies per cell. According to the orthodox mtDNA replication model (Shadel and Clayton, 1997), replication is initiated at two origins, one in each strand (O_H and O_L , for the H- and L-strands, respectively). Replication of mtDNA is unidirectional and asynchronous, starting at O_H located adjacent to the D-loop and traversing two thirds of the genome until O_L is exposed, after which L-strand replication starts in the opposite direction. This mechanism does not, however, alone account for human mtDNA replication in all circumstances, but unidirectional coupled leading- and lagging- strand synthesis is also in operation (Holt et al., 2000). mtDNA is transcribed in multicistronic transcripts from two promoters in the H-strand and one in the L-strand, and the primary transcript is subsequently cleaved to generate RNAs for individual genes (Reviewed in: Clayton, 1992).

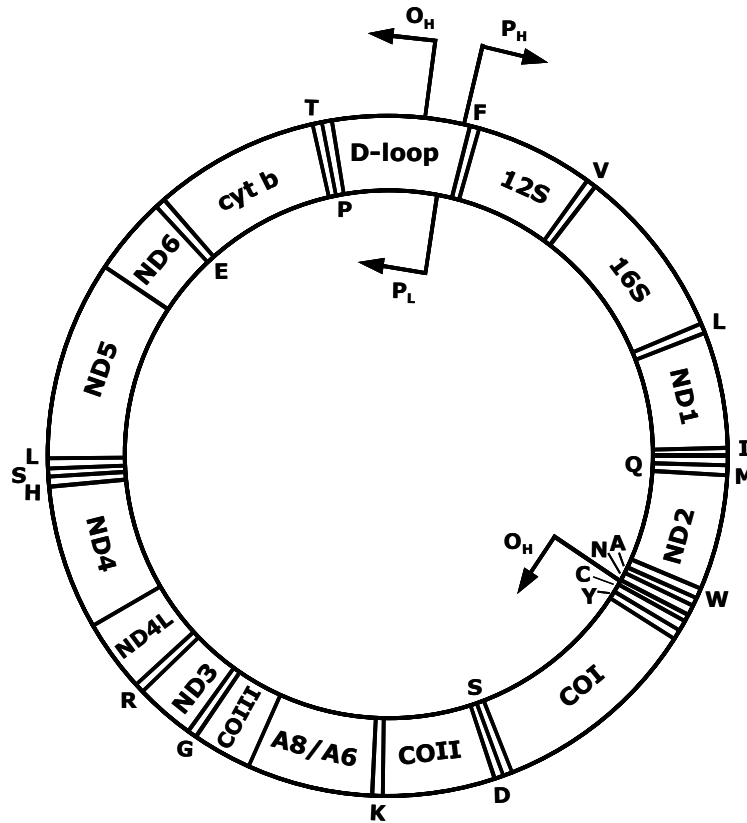


Figure 1. Human mtDNA map. O_H = H-strand replication origin, O_L = L-strand replication origin, P_H = H-strand promoter, P_L = L-strand promoter. Abbreviations for gene names: 12S and 16S = 12S and 16S ribosomal RNA genes, ND1-6 and ND4L = NADH dehydrogenase 1-6 and 4L, CO I-III = cytochrome c oxidase subunits I-III, A6 and A8 = A6 and A8 subunits of ATP synthase, cyt b = cytochrome *b*. tRNA genes are given as the one-letter amino-acid code.

6.1 Manifestation of mtDNA disorders in heart

Together with disorders of myocardial contractile and structural proteins, the disorders of cardiac energy metabolism are the major cause of primary cardiomyopathy (Kelly and Strauss, 1994). Abnormalities of cardiac energy metabolism include defects of OXPHOS and defects of β -oxidation. OXPHOS defects can be genetically classified as large-scale rearrangements of mtDNA, point mutations of mtDNA and traits with Mendelian inheritance (Antozzi and Zeviani, 1997). Hypertrophic cardiomyopathy has been shown to be caused by mtDNA point mutations (Casali et al., 1995, Zeviani et al., 1995) and cardiomyopathy is one of the common features of syndromic diseases involving mtDNA deletions (Bohlega et al., 1996). Rare cases of dilated cardiomyopathy due to mtDNA deletions have also been reported (Suomalainen et al., 1992, Moslemi et al., 2000).

Clinically, large-scale mtDNA rearrangements are expressed as Kearns-Sayre syndrome (KSS), Pearson's syndrome and sporadic progressive external opthalmoplegia (PEO), which are multisystem diseases involving most often a single mtDNA deletion.

Cardiomyopathy is unusual in KSS, but deterioration of heart conduction, manifested as various branch blocks, atrioventricular or complete heart block have often been seen (Roberts et al., 1979, Remes et al., 1992). PEO with autosomal dominant (ad) or autosomal recessive (ar) inheritance and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), both present with multiple, differently sized mtDNA deletions. Ar-PEO with multiple deletions and childhood onset has been described to cause severe cardiomyopathy (Bohlega et al., 1996), but autosomal dominant PEO (Zeviani et al., 1989, Moslemi et al., 1999) or MNGIE (Hirano et al., 1994) rarely involve the heart. Friedreich's ataxia (FRDA) has been recently recognized to be caused by mutations in the gene for frataxin, a mitochondrially located nuclear DNA-encoded protein, the deficiency of which causes mitochondrial iron accumulation and probably increased oxidative stress (Reviewed in: Puccio and Koenig, 2000). Although a mainly neurodegenerative disease, FRDA often involves heart in the form of hypertrophic cardiomyopathy, which can progress to a dilated form (Casazza and Morpurgo, 1996).

Isolated cases of idiopathic cardiomyopathies have recently also been claimed to be associated with mtDNA rearrangements present at low levels (Ozawa et al., 1990, Bobba et al., 1995, Li et al., 1995, Marin-Garcia et al., 1996, Ruppert et al., 2000). The pathological relevance of these reported mtDNA alterations has not been evidenced, and the quantitative significance of the small amounts of aberrant molecules has been challenged further by others, who have shown them in comparable abundance in myocardium of controls (Remes et al., 1994, Lightowlers et al., 1999). The prevalence of the most common pathogenic mtDNA point mutations seems to be extremely low among unselected idiopathic cardiomyopathy patients (Turner et al., 1997). At present the association of any of the various heterogeneous mtDNA rearrangements with myocardial disease can not be fully excluded and new data are needed.

Animal models have shed some light into the functional consequences of mitochondrial dysfunction *in vivo*. Disruption of the mouse gene for mitochondrial transcription factor A (Tfam) led to severe mtDNA depletion and embryonic lethality (Larsson et al., 1998). Inactivation of Tfam in a heart- and skeletal muscle -specific way caused a mosaic pattern of respiratory chain deficit in the heart plus dilated cardiomyopathy and atrioventricular conduction block, thus reproducing the essential phenotypic features of KSS (Wang et al., 1999). Mice lacking adenine nucleotide translocator 1 (Ant1), which is the heart, muscle and brain -specific isoform of the mitochondrial inner membrane transporter, developed features characteristic of mitochondrial disease, including proliferation of mitochondria in the muscle, cardiac hypertrophy, exercise intolerance and a global metabolic acidosis (Graham et al., 1997). Inactivation of the mouse gene *Frda* (homolog of the Friedreich's ataxia disease gene in humans) caused early embryonic lethality (Cossee et al., 2000). Further experiments using tissue-targeted gene disruption showed the pathophysiological and biochemical features of the human disease, including cardiac hypertrophy, degeneration of certain type of sensory neurons, reduced activities of complexes I-III and intramitochondrial iron accumulation (Puccio et al., 2001). Manganese superoxide dismutase (MnSOD) knockout mice present with a dilated cardiomyopathy, accumulation of

lipid in liver and skeletal muscle and metabolic acidosis with complex II deficiency (Li et al., 1995).

6.2 Methods used to study mtDNA rearrangements

The standard method for detection of mutant mtDNAs with gross rearrangements has been Southern blotting and hybridization with a mtDNA probe. The fraction of aberrant mtDNA forms (in states involving a single clonal deletion) or various forms (in multiple deletion disorders) are quantified as the ratio of their hybridization signal to that of the wild-type mtDNA. More indirect methods, involving PCR amplification across the deletion breakpoints and quantification of the deleted species using an internal PCR standard, have been used to study small amount of deletions e.g. in aged tissues (Cortopassi and Arnheim, 1990). As these methods analyze only one deletion at the time, a derivative of standard PCR, called long-extension PCR (LX-PCR), has been recruited (Li et al., 1995, Melov et al., 1995). LX-PCR primers can be designed to amplify several kilobases or the whole 16.6 kb mtDNA genome, allowing detection of nearly all theoretical mtDNA rearrangements in one reaction.

The use of transmitochondrial cybrid cell lines, made by fusing enucleated donor cells with cells depleted of mtDNA by ethidium bromide treatment (ρ^0 -cells), has become a standard method to study functional consequences of mtDNA mutations (King and Attardi, 1989). MtDNA from patients with pathological deletions has been introduced into cell lines (Hayashi et al., 1991a). By fusion of aged mouse synaptosomes with ρ^0 -cells it has been possible to trap deletion mutants of mtDNA (Inoue et al., 1997), and subsequently to generate transgenic mice carrying a mtDNA deletion in their tissues (Inoue et al., 2000).

6.3 What causes pathological mtDNA rearrangements?

Partially duplicated mtDNAs have been reported to be maternally transmitted (Rotig et al., 1992, Dunbar et al., 1993), and to yield the molecular species detected in the offspring, probably by generation of deleted molecules from partial duplications by homologous recombination. At least in some nuclear backgrounds, cell clones having 100 % partially duplicated mtDNA molecules have have given rise to wild-type mtdna during culture, strongly suggesting that intramolecular homologous recombination had taken place (Holt et al., 1997, Tang et al., 2000). Intermolecular recombination events, i.e. reverse reactions creating duplicated molecules from wild-type and deleted species, would be required to explain the existence of duplicated mtDNA molecules in the first place. This could occur by at least two different mechanisms: (1) (intermolecular) recombination between two wild-type mtDNAs to give a dimer, which then could create both the partially duplicated and the deleted forms by intramolecular recombination. (2) intramolecular recombination of wild-type mtDNA to give the deleted form, followed by an intermolecular recombination event to create the partially duplicated form. Partial duplications could also arise by a mechanism that does not involve intermolecular recombination, if replication continued past the normal termination site, to create a dimer. There is no data supporting the creation of partially duplicated mtDNA

molecules from wild-type and deleted molecules. However, on rare occasions, a partially triplicated form of mtDNA has been detected in long-term culture of a cell line originally containing only partially duplicated molecules, supporting intermolecular recombination (Holt et al., 1997). Other possible mechanisms generating rearrangements have also been suggested, such as slipped mispairing during replication (Shoffner et al., 1989, Madsen et al., 1993). In addition, in theory a hypothetical rolling-circle –like replication mechanism could also create the partially duplicated mtDNA molecules.

Great majority of the disorders with mtDNA rearrangements occur either sporadically or display autosomal dominant (ad) or autosomal recessive (ar) modes of inheritance. MNGIE, a disorder with multiple deletions and/or partial depletion of mtDNA was recently shown to be caused by mutations in the gene for thymidine phosphorylase, an enzyme involved in nucleotide metabolism (Nishino et al., 1999). The activity of this enzyme in patients was less than 5% of controls, and this defect in thymidine metabolism may impair mtDNA replication, maintenance or both (Nishino et al., 1999). Ad-PEO, another disorder with multiple mtDNA deletions and Mendelian inheritance, was recently reported to be caused by heterozygous mutations in the heart/skeletal muscle isoform of adenine nucleotide translocator (ANT1) (Kaukonen et al., 2000). ANT homodimers are known to form a gated pore through which ADP is moved from the mitochondrial matrix to the cytosol, but its exact role in mtDNA maintenance is still obscure (Kaukonen et al., 2000).

6.4 mtDNA and theories for aging and neurodegenerative diseases

The mitochondrial theory of aging states that cell aging is the result of bioenergetic decline caused by accumulation of somatic mutations in mtDNA (Harman, 1981, Fleming et al., 1982). It has been hypothesized that since mtDNA lies in the immediate vicinity of the free radical -producing respiratory chain, and does not have the full repertoire of DNA repair mechanisms as does the nucleus (Clayton et al., 1974), the rate of mutagenesis would be high (Harman, 1981, Fleming et al., 1982). More recently it has been shown that several systems for DNA repair do function effectively also in mitochondria (Pettepher et al., 1991, Driggers et al., 1993). Based on results obtained by PCR, some authors have calculated that in high-energy tissues of 70-year old individuals there would be ca. 50 base substitutions per mtDNA molecule (Nagley and Wei, 1998). Paradoxically, another study sequencing clonal PCR products did not reveal a single mutation outside the highly variable noncoding D-loop region, even in 94 and 99 year-old individuals studied (Jazin et al., 1996). There is also much controversy about the accumulation of deletion mutations during aging (Lightowlers et al., 1999). The so called common 4977 bp deletion has been reported to accumulate with age in different regions of brain up to 2000-fold (Corral-Debrinski et al., 1992). A specific point-mutation has also been suggested to accumulate in the extraocular muscles of the elderly to heteroplasmy levels of ca. 2 % of the total mtDNA (Munscher et al., 1993). In contrast to the proposed high mutation rate of mtDNA in general, small insertions and deletions have been reported to accumulate at much lower rates (to less than 0.001% of mtDNA), and point mutations may not accumulate at all (Jazin et al., 1996). In an cell-line model of proof-reading

deficient human DNA polymerase γ (POLG), a high mutational load of on average 10 base substitutions per mtDNA molecule had little or no phenotypic effects on cellular respiration (Spelbrink et al., 2000).

Bioenergetic decline during aging as a universal phenomenon has been routinely used as evidence for the mitochondrial theory of aging (Shigenaga et al., 1994, Melov et al., 1995). The function of respiratory complexes was initially reported to be impaired during aging (Trounce et al., 1989, Yen et al., 1989), but this has been questioned in more recent studies showing no correlation between age and respiratory decline (Barrientos et al., 1996, Brierly et al., 1997, Chretien et al., 1998). Functional testing of mitochondria, carried out using the *rho*⁰ *trans*mitochondrial system, revealed modest changes in cybrid oxygen consumption by age (Laderman et al., 1996). The interindividual differences in respiration capacity however exceeded clearly the small decrease occurring over the whole lifespan (Laderman et al., 1996), which suggests that a causal relationship with aging is unlikely. Furthermore, other studies similarly using cybrid techniques have concluded that aging-related decline in OXPHOS of human skin fibroblasts was due to nuclear instead of mtDNA –derived factors (Hayashi et al., 1994, Isobe et al., 1998).

Many reports and reviews have suggested the involvement of decreased mitochondrial respiration capacity in development of age-related neurodegenerative diseases (e.g. Linnane et al., 1989, Wallace, 1992). Both somatic and maternally inherited mtDNA mutations have been implicated to be causal in the process leading to disease. Some studies have observed Alzheimer's and Parkinson's disease –derived mitochondria to display cytochrome *c* oxidase (COX) (Davis et al., 1997, Sheehan et al., 1997) or complex I (Swerdlow et al., 1996) deficiency. Recently, however, it was shown that mitochondria from donor brains of elderly Alzheimer's disease (AD) patients showed respiratory function comparable to age-matched controls, suggesting functional integrity of mtDNA (Ito et al., 1999). One report suggested that mtDNA mutations might cause as much as 20% of all AD cases (Davis et al., 1997). Surprisingly, another study found these mutations to be very rare (2-3%) and comparable to controls in brain of a large group of AD patients (Hutchin et al., 1997). The results of Davis et al. (1997) were questioned even further by showing that the analyses were most probably confounded by nuclear pseudogenes (Hirano et al., 1997, Wallace et al., 1997). It has also been pointed out that if certain mtDNA mutations would be causative of AD, most late-onset AD cases should be inherited maternally and mitochondrial diseases should express phenotypes related to AD, neither of which is not the rule (Ito et al., 1999), although MERRF and KSS patients do display dementia. If AD causing mutations would be considered to arise somatically during aging, mitotic tissues like platelets (Davis et al., 1997, Sheehan et al., 1997), should in any case not accumulate them because of mitotic segregation of mutant mtDNAs combined with selection (Lightowlers et al., 1997).

Two major arguments have been used to ascribe significance to the low amounts of mtDNA mutations claimed to accumulate in tissues, which are held to contribute to cellular aging and neurodegenerative diseases. Firstly, the 'tip of the iceberg' hypothesis proposes that since the total number of different defective mtDNA species is very high, all of the aberrant forms of mtDNA cannot be studied quantitatively in a single assay, hence the total mutational

load must be underestimated. However, in the original LX-PCR paper (Melov et al., 1995), Southern blotting analyses showed strong signal from wild-type mtDNA in aged specimens, which would most probably not be the case if most of the mtDNA molecules bear rearrangements. Secondly, it has been proposed that mutant mtDNAs, although at low abundance in a given tissue as a whole, put forward can be concentrated in certain cells or cell-types, in which functionally significant levels of mutations would be reached. In muscle of aged individuals, a small number of COX negative fiber segments have been observed, and these have been associated with mutated mtDNA (Muller-Hocker et al., 1993, Brierley et al., 1998). However, if matching for physical activity is done, there has been little correlation between oxidative metabolism and age (Brierley et al., 1996).

7. Genetics of AHMD

Only a minority (ca. 1-5 %) of alcoholics ever develops a fulminant heart muscle disease, while most do not even after decades of very heavy alcohol use. Although environmental factors, nutrition, infections, and concomitant other diseases explain only part of individual susceptibility, the issue of genetic risk factors has not yet been elucidated. Of the other alcohol-related traits, alcoholic liver disease has been agreed to have a considerable genetic background (Hrubec et al., 1981), but the individual genes involved have not yet been identified. Difficulties in assessing the genetic susceptibility loci to AHMD arise from incomplete data on the dose-dependency of the cardiac abnormalities and the lack of agreement on the specific, if any, myocardial changes to define the AHMD phenotype reliably. Therefore, studies concerning the genetic background of AHMD should first define the phenotype and importantly control for confounding factors, such as coronary heart disease.

7.1 Renin-angiotensin-aldosterone –system

Aldosterone controls the sodium balance and water retention in the body, taking part also in the regulation of blood pressure. The secretion of aldosterone is mainly controlled by the renin-angiotensin system (for review see White, 1994). When intravascular volume is decreased, the juxtaglomerular cells of the kidney secrete renin, which converts the precursor angiotensinogen into angiotensin I, which is then converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II then acts on the adrenal zona glomerulosa and increases the activity of aldosterone synthase and thus aldosterone production and secretion. Angiotensin II is involved also in other pathways, having e.g. a vasoconstrictive effect of smooth muscle cells via angiotensin II type 1 receptor (AT1R).

Several studies have suggested that genetic variation in the enzymes of RAA –system might predict LV mass and/or function of healthy individuals, as well as of patients having cardiovascular disease. The insertion/deletion (I/D) polymorphism of the ACE gene is associated with serum ACE levels (Rigat et al., 1990). Contradictory results have been

published on its effect of left ventricular mass, showing either an increase (Iwai et al., 1994, Schunkert et al., 1994,) or no effect (Kupari et al., 1994, Lindpaintner et al., 1996). The *AT1R* 1166A/C polymorphism is associated with response to angiotensin II in human arteries (Amant et al., 1997, Henrion et al., 1998, van Geel et al., 2000). This polymorphism has been associated with left ventricular function (Hamon et al., 1997, Reissell et al., 1999) and cardiac hypertrophy in cardiomyopathic patients (Osterop et al., 1998), but it did not modulate the physiological responses to angiotensin II infusion in young volunteers (Hilgers et al., 1999). Aldosterone synthase gene (*CYP11B2*) –344C/T polymorphism has been shown to a certain extent to modulate the activity of the renin-angiotensin system (Hautanen et al., 1998, Pojoga et al., 1998, Davies et al., 1999, Paillard et al., 1999). Among normal subjects the urinary secretion rate of aldosterone or its metabolites has been elevated for the T allele (Hautanen et al., 1998, Davies et al., 1999, Paillard et al., 1999), lacking however any clear gene-dosage effect. In young, healthy volunteers the –344C/T polymorphism associated strongly with left ventricular dimensions, mass and diastolic filling (Kupari et al., 1998). A larger and more heterogeneous material from the echocardiographic substudy of the third MONICA (Schunkert et al., 1999) or a sample of patients with myocardial infarction (Hengstenberg et al., 2000) did not show any correlation with –344C/T genotype and LV measurements or blood pressure. Neither were serum aldosterone levels dependent on the genotype (Schunkert et al., 1999).

7.2 Enzymes of ethanol metabolizing pathways

The chromosomal loci for human ADH2 code for 3 different allelic subunits or isoforms (β 1- β 3). The alleles of ADH2 possess marked differences in their enzymatic function, the ‘mutant’ β 2 and β 3 subunits (coded by alleles *ADH2:2* and *ADH2:3*) oxidizing ethanol 30-40 times faster than the common β 1 subunit (Bosron and Li, 1986, Thomasson et al., 1995). The ‘fast’ alleles of ADH2 are very rare in Caucasians, but common in Oriental populations (Goedde et al., 1992, Gilder et al., 1993). It has been hypothesized that individuals with β 2 or β 3 alleles could be more vulnerable to the deleterious effects of ethanol because of faster acetaldehyde production and possible accumulation. ADH3 is expressed as 2 different allelic subunits (γ 1 and γ 2), of which the γ 1 subunit (*ADH3:1* allele) is three times faster in ethanol oxidation compared to the common γ 2 subunit (*ADH3:2* allele) (Bosron and Li, 1986). The *ADH3:1* allele is a common variant also in Caucasian populations.

The human mitochondrial ALDH2 is responsible for the majority of acetaldehyde elimination. ALDH2 exists in two allelic forms, ALDH2:1 and ALDH2:2, which differ with respect to their enzymatic activities. Allele 2 homozygotes have only little or no catalytic activity and heterozygotes about half the activity of the *ALDH2:1* homozygotes. (Crabb et al., 1989) While in Oriental populations the inactive ALDH2*2 allele is frequent, it is very rare in Caucasian populations (Goedde et al., 1992).

CYP2E1 also contains several polymorphic sites, of which the *Pst*I and *Rsa*I polymorphisms have been associated with alcoholic liver disease (Maezawa et al., 1994).

While these genetic variants are common in Oriental populations, they are almost absent in Caucasians, like Finns (Hirvonen et al., 1993). *DraI* and *MspI* polymorphisms of *CYP2E1* are of unknown functional significance but *DraI* polymorphism was linked up with the prevalence of alcoholism in one study (Lucas et al., 1996). The *DraI* locus is polymorphic also among Finnish population (Hirvonen et al., 1993).

AIMS OF THE STUDY

The aim of the present study was to clarify the relationship between alcohol consumption and cardiac remodeling, as well as to evaluate possible risk factors of alcoholic heart muscle disease (AHMD). More specifically, the aims of this study were defined as follows:

1. To study the relationships between alcohol intake, cardiac anatomy and AHMD.
2. To study the possible effects of cardiovascular risk factors and concomitant coronary artery disease on the development of AHMD.
3. To study the involvement of mitochondrial DNA (mtDNA) re-arrangements in alcohol-related heart pathologies and to test candidate genetic variants possibly predisposing to AHMD.

MATERIALS AND METHODS

1. The autopsy series

Our study material consists of two series of autopsies conducted at the Department of Forensic Medicine, University of Helsinki. The first of these (series A), 400 consecutive males, was collected during 16 months in 1981-82 and the second (series B), 300 consecutive males, during 12 months in 1991-92. This series of altogether 700 autopsies is a part of Helsinki Sudden Death Study, which was launched in the early 1980's as a complementary independent study to the WHO MONICA –project (Tunstall-Pedoe, 1985, Tunstall-Pedoe et al., 1999) to characterize the risk factors and pathology of sudden cardiovascular deaths. Male sex and suitability for a complete autopsy were used as inclusion criteria. In addition, the very young and old victims were excluded, making the final study series consist of males aged 33-70 years, covering 42 % of all deaths of individuals <65 years in the area of Helsinki city and its surroundings during the study periods. Among the 700 cases, the cause of death was cardiovascular in 290 (41.4 %), other disease in 130 (18.6 %), intoxication in 138 (19.7 %), violence in 134 (19.1 %) and remained unknown in 8 (1.1 %) cases. This autopsy series has previously been used to study alcoholic liver disease (Savolainen et al., 1993) and alcohol-related disorders of spermatogenesis (Pajarinen et al., 1997).

2. Evaluation of alcohol consumption and other cardiovascular risk factors

A structured interview of the deceased's spouse or a close relative or acquaintance was used to evaluate the lifetime habitual alcohol consumption. The interview was usually carried out, personally, within 2 weeks of death by the pathologist who performed the autopsy. Out of the 700 consecutive cases from the two time periods, a person with sufficiently detailed knowledge of the deceased's lifetime history could be reached for interview in 500. The questionnaire incorporated 14 items concerning the intensity, frequency, quantity and quality of the individual's alcohol consumption. (Karhunen and Penttilä, 1990) Questions were similar to those in AUDIT (Fleming et al., 1991) and MAST (Selzer et al., 1968). The responses were used to calculate an estimate of all-year average daily alcohol intake as absolute ethanol (grams/day). Such an estimate of alcohol consumption could be obtained for 452 men of the whole series. In the remaining 48 cases interpretation of the interview data was difficult because of one or more of the following reasons: only a distant acquaintance was reached for interview, there was conflict between the interview data and data from other sources (police and hospital records), in the control or moderate groups there were subjects with suspected alcoholism or high blood or urine alcohol at autopsy or the subject had episodic drinking periods. In addition, 17 of the 452 men were former heavy drinkers who had recently reduced their drinking markedly. A change, which shifted the subject from one consumption group (see next sentence) to another, was considered marked. Thus, we had 435

men with stable drinking habits for our analyses. To be used in some of the analyses, these men were classified according to their daily alcohol dose either as teetotalers or light drinkers (<12 g/day = <1 drink), moderate drinkers (12-72 g/day = 1-6 drinks), heavy drinkers (72-180 g/day = 6-15 drinks) or very heavy drinkers (>180 g/day = >15 drinks).

The interview included also questions regarding smoking and other cardiovascular risk factors. We recorded the average daily cigarette consumption as well as the number of smoking years and whether the deceased was an ex-smoker. Whether hypertension or diabetes had been diagnosed in the past was also specifically questioned and recorded. In 223 cases, the history of these diseases remained unknown, mostly because the deceased was not known to have visited a doctor.

3. Quantification of coronary artery disease and diagnosis of myocardial infarction

The epicardial coronary arteries were evaluated first by making a silicone rubber cast of the entire coronary artery tree (for the detailed method, see Weman et al., 1999). Essentially, ascending thoracic aorta was transected, and the contrast medium (silicone rubber made radiopaque with lead oxide) container connected by a plastic mouthpiece to the vessel. The contrast medium was then delivered to the circulation by applying a physiological pressure (120-180 mmHg) for 30 minutes. Another 2-3 hours were given to the medium to solidify properly. Then the coronary arteries were longitudinally opened and the cast model removed and filed for later analyses. In the analysis, any local narrowing of the lumen of the coronary arteries was measured and determined as a percentage of the most adjacent uninvolved lumen.

The presence of myocardial infarction was documented macroscopically by nitrobluetetrazolium (NBT) staining and confirmed by a histologic examination of the myocardium.

4. Measurement of ventricular dimensions and weights

The basis of the study methodology was formed by a complete routine medicolegal autopsy, to which cardiac examinations for the purposes of this study were added. The heart was weighed and the ventricular block was cut into 15-mm transversal slices after the examination of the coronary arteries. The borders of the LV and RV cavities and walls at the equatorial region were traced onto a transparent sheet for later analyses. The muscle of left and right ventricle was weighed separately by collecting all of the ventricular muscle pieces dissected free of epicardial fat. The interventricular septum was included in the LV weight. The traced anatomic areas of the ventricles and the wall thicknesses were measured later by routine computer-assisted planimetry methodology. (Figure 1) The postmortem delay before autopsy was on average 3.6 days (median 3 days), but did not correlate with any of the ventricular dimensions or weights.

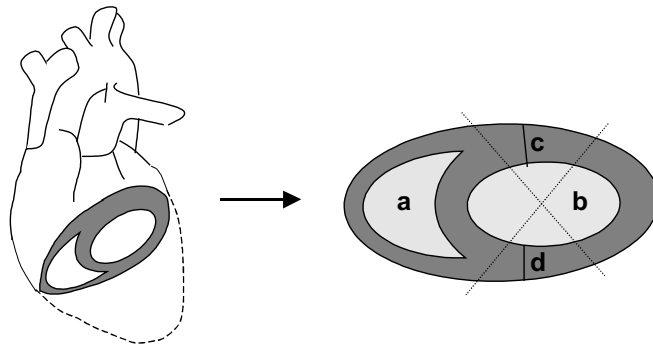


Figure 2. A cartoon schematically showing the variables measured by planimetry. a=right ventricle, b=left ventricle (LV), c=LV anterior wall, d=LV posterior wall. c and d were measured inside the area between the dashed lines.

5. mtDNA rearrangements

5.1. Patients and Tissue Samples

Frozen samples of heart muscle were obtained from the series of 300 medico-legal autopsies performed in 1991-92 (series B). For the study III, 8 cases of autopsy verified alcoholic cardiomyopathy (ACM) plus 10 controls without myocardial disease or heavy alcohol intake were analyzed. Paediatric biopsy and autopsy samples (11 skeletal muscle, 4 heart, 2 liver, 1 brain from children from 14 weeks gestation to 14 years) were collected in the John Radcliffe Hospital, Oxford and Tampere University Hospital, respectively, from individuals without diagnosed mitochondrial or other relevant systemic disease. Muscle biopsies from 6 adults (ages 18-82), from whom no mitochondrial disease was found in the studies, were taken at the Neurology clinic in University of Oulu or in Oxford. *Post mortem* tissue samples (time from death to autopsy 2-3 days) were taken from each of 6 other individuals (s1-s6) during the routine autopsy protocol (see study V) and the DNA was extracted immediately after sampling. Control sperm samples from anonymous males known to have fathered children were kindly provided by Dr L. Wichmann, Department of Anatomy, University of Tampere.

5.2. Cell lines

143B osteosarcoma-derived and A549 lung carcinoma-derived *rho*⁰ cells (lines 206 and B2, respectively, see King and Attardi, 1989, Bodnar et al., 1993) were cultured in Dulbecco-modified Eagle's medium/10% fetal bovine serum, supplemented with pyruvate and uridine (Bodnar et al., 1993).

5.3. DNA extraction

Total DNA was extracted and purified from the frozen heart samples by standard methods at the Department of Forensic Medicine, University of Helsinki. According to the basic protocol, a frozen piece of the autopsied heart muscle (1-5 grams, -70 °C) was first cut at -20 °C into thin slices with a sterile disposable scalpel. These roughly homogenized tissue samples were dissolved in 2 ml of proteinase K -buffer (containing 25 mM EDTA, pH 8.0 and 75 mM NaCl). 0.5 mg (0.5 ml) of proteinase K and 0.1 ml of 20 % SDS were then added and the solution was incubated overnight at 37 °C. The samples were then pipetted into Phase Lock Gel II B Light -tubes (5-prime-3-prime, USA) prepared according to the manufactures's instructions. 4 ml of phenol/chloroform/isoamylalcohol (25:24:1) was then added, the tubes were shaken well and centrifuged at 2000 rpm for 5 min. The aqueous phase from this extraction plus 0.2 ml of 2 M KCl were transferred to a sterile tube and shaken gently. DNA was precipitated by adding 5 ml of ice-cold 80 % ethanol. The precipitated DNA was collected with a closed Pasteur pipette, air-dried and redissolved in 0.5 ml of TE-buffer. DNA concentration was measured spectrophotometrically.

5.4. Long extension PCR (LX-PCR)

By long extension PCR (LX-PCR) the full mitochondrial genome was amplified using two different sets of primers (Figures 2 and 3, Tables 1 and 2), one in the non-coding D-loop area, the other inside the gene for 16S rRNA. Several overlapping subgenomic mtDNA fragments were amplified in other LX-PCR reactions (Figures 2 and 3, Tables 1 and 2). DNA polymerase used in all the reactions was DyNAzyme EXT (Finnzymes OY, Espoo, Finland), which is suitable for long PCR amplifications and has weak 3' → 5' proofreading and 5' → 3' exonuclease activities. A hot start was performed by dividing the PCR mix into two phases by Dynawax (Finnzymes), which melts at high temperature allowing the PCR reaction to start. Thin-walled 0.5 and 0.2 ml tubes (Robbins Scientific Corp., USA) and a PTC-100 (MJ Research Inc., USA) or GeneAmp PCR System 9700 (Perkin Elmer, USA) thermocycler was used. The PCR reaction (50 µl) consisted of 0.6 µM of each primer, 500 µM each dNTP, 1 X EXT buffer supplied with the enzyme (50 mM Tris-HCl, pH 9.0 at 25 °C, 15 mM (NH₄)₂SO₄, 0.1 % Triton X-100), 2.5 mM MgCl₂, 2 % DMSO, 1 unit EXT DNA polymerase and template DNA. An initial denaturation of 2 min was followed by 10 cycles of (denaturation, annealing, primer extension) (30 sec at 93 °C, 1 min at 60 °C, 12 min at 68 °C). During the following 20 cycles, the primer extension time was increased by 20 sec per cycle and a final extension was performed for 7 min at 68 °C. The PCR products were separated on standard 0.7 % agarose gels, stained with ethidium bromide and visualized under UV-illumination. The LX-PCR products were southern blotted and hybridized as described in 5.5. The oligonucleotide primers for PCR were purchased from DNA Technology (Aarhus, Denmark) or Genset (Paris, France).

5.5. Southern blotting

Five or 10 µg of genomic DNA, with or without *Bam*HI digestion, were fractionated by 0.7 % agarose gel electrophoresis in the absence of ethidium bromide. The gel was then soaked for 10 min in 0.25 M HCl to facilitate the transfer of large DNA fragments, twice for 20 min in 0.5 M NaOH/1.5 M NaCl and twice for 20 min in 0.5 M Tris-HCl/1.5 M NaCl, pH 7.4. DNA was transferred overnight to a MagnaCharge nylon membrane (Micron Separation Inc., Westborough, USA), washed lightly for 5 min in 4X SSC and UV-linked. Prior to hybridization, the filter was pre-wetted in 4X SSC, 0.1 % SDS for 20 min and prehybridized 1 hour at 50 °C in the hybridization solution (2.5 % dextran sulfate, 0.1 mg/ml denatured, sonicated salmon sperm DNA, 0.025 sodium phosphate buffer, pH 6.8 (PB), all in 4X SSC). Hybridization was carried out at 68 °C for 15 hours. Stringent washes of the blot were carried out in solutions prewarmed to 68 °C: one quick wash plus 20 min in 5X SSC, 0.1% SDS, 0.025 M PB, twice for 20 min in 1X SSC, 0.1% SDS, 0.025 M PB and twice for 20 min at 0.2X SSC, 0.1 % SDS, 0.025 M PB. The radioactive hybridization signal was detected by autoradiography.

The probes for southern blots were generated by PCR from DNA template of one of the control heart muscle samples. 690 bp and 717 bp fragments were synthesized, respectively from 16S/ND1 (by primers FR6H/FR7L: see table 1) and ND4/ND5 (by primers FR51H/FR44L). The specific PCR products were purified from agarose with Wizard PCR preps (Promega). These fragments were labelled by nick translation using Oligolabelling Kit (Pharmacia Biotech) and [α -³²P]dCTP (Amersham, 3000 Ci/mmol).

The sublimon junctional oligonucleotide Junc-1 (GGTAATCGCATAAAACCA-TCAACAACCGCT, np 3247-3261 + 16072-16086 of human mtDNA) was end-labeled with [γ -³²P]ATP (6000 Ci/mmol, Amersham) using T4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania) in the supplied reaction buffer A, according to the manufacturer's instructions, before addition to the hybridization reaction. Hybridization was carried out at 50 °C for 16 hours after a pre-hybridization step of 1 hour at 50 °C without the probe. The blot was washed for 10 min in 5x SSC, 0,1 % SDS, 0.025 M PB and for 5 min in 1x SSC, 0,1 % SDS, 0.025 M PB, both at 50 °C. The filters were stripped in boiling 0.5 % SDS and reprobbed with a probe for the ND4/ND5 or 16S/ND1 region of human mtDNA.

5.6 Molecular cloning and sequencing

LX-PCR reactions were carried out using the 'genome length' primer pair OK1H/OK2L and for cloning of the largest sublimons (PCR products in the range of 6-15 kb) the 'sub-genomic' primer pair FR31H/OK2L. 26 ng of a control heart DNA template (individual BT91, 49 year-old male) that had given diverse and abundant sublimon products was used. The LX-PCR products were cloned into pCR-XL-TOPO-vector (Invitrogen BV, Groningen, Netherlands) according to the manufacturer's instructions. In all 65 clones (42 obtained by PCR using 'genome length' primers, plus 23 derived using 'sub-genomic'

primers) were selected, sized by miniprep analysis and sequenced using the BigDye terminator kit (Applied Biosystems, Foster City, CA), with a combination of vector-specific and mtDNA-specific primers. The sequencing reaction products were analysed by capillary electrophoresis on an Applied Biosystems 310 Genetic Analyzer, using the manufacturer's software.

Table 1. Oligonucleotide Primers for LX-PCR

Oligo-nucleotide	Sequence 5' → 3'	5' co-ordinate [#]	Location
OK1H	TCGCACCTACGTTCAATATTACAGGCGAAC	160	ncr
OK2L	TAAATAATAGGATGAGGCAGGAATCAAAGACA	161	ncr
FR6H	GGTGCAGCCGCTATTAAAGGTTTCGT	3012	16S
OK6L	ATCGGGATGTCCTGATCCAACATCG	3013	16S
FR7L	CCGATCAGGGCGTAGTTTGGAGTTTG	3698	ND1
FR31H	CTTCCCACAACACTTTCTCGGCCTA	7178	COI
FR32L	GTAAAGGATGCGTAGGGATGGGAGG	7840	COII
FR51H	ACTTCTAGCAAGCCTCGCTAACCTC	11836	ND4
FR44L	TTTGGGTTGTGGCTCAGTGTCAGTT	12553	ND5

[#] According to Anderson et al., 1981

Table 2: Predicted PCR products synthesized by LX-PCR

Primer pair	Sequence amplified	Length of product (bp)	Product ID [#]
OK1H→OK2L	161-160	16568	ns
FR6H→OK6L	3013-3012	16568	ns
FR6H→OK2L	3013-160	13716	7
FR31H→FR7L	7178-3698	13089	ns
FR51H→FR32L	11836-7840	12573	ns
OK1H→FR44L	161-12553	12392	6
FR31H→OK2L	7178-160	9551	5
FR6H→FR44L	3013-12553	9540	4
FR51H→FR7L	11836-3698	8431	3
OK1H→FR32L	161-7840	7679	2
FR31H→FR44L	7178-12553	5415	1
FR6H→FR32L	3013-7840	4827	ns

[#] As denoted on Fig. 4. ns = not shown on Fig. 4

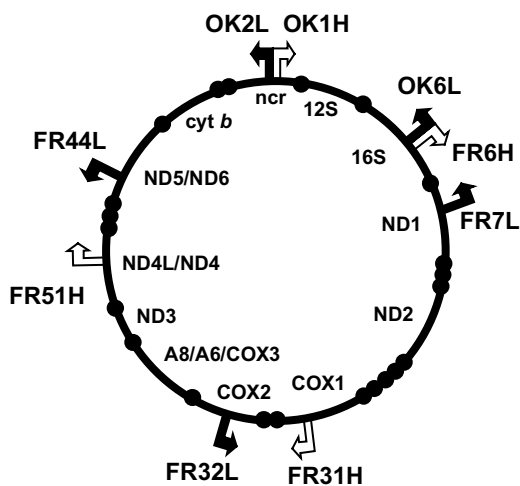


Figure 3. Schematic mtDNA function location map showing primers used for LX-PCR. ncr = non coding region, 12S and 16S = 12S and 16S ribosomal RNA genes, ND1-6 and ND4L= NADPH dehydrogenase 1-6 and 4L, COX 1, 2 and 3 = cytochrome oxidase 1, 2 and 3, A6 and A8 = A6 and A8 subunits of ATP synthase, cyt b = cytochrome b. Filled circles = tRNA genes.

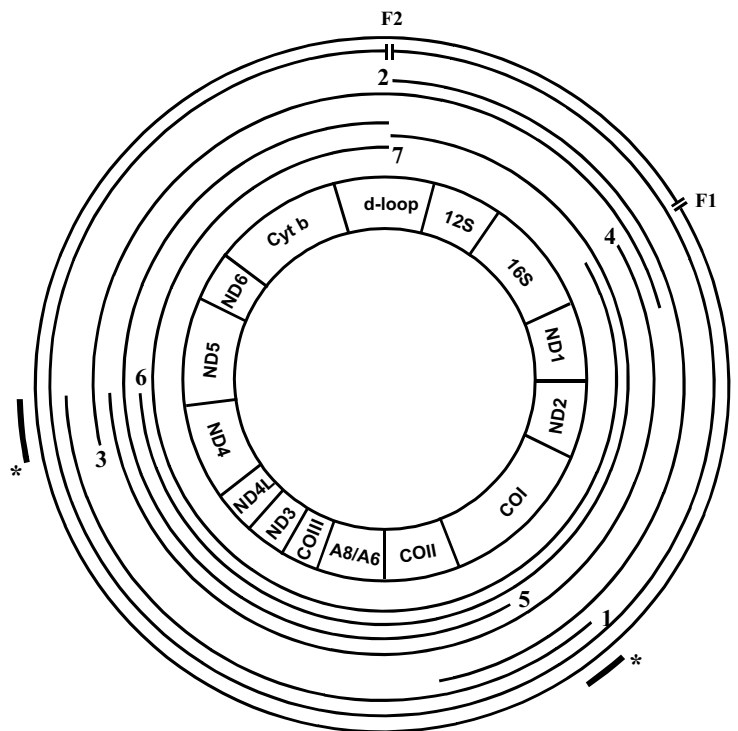


Figure 4. Location of the predicted LX-PCR products in mtDNA. Numbering of products is analogous to the Table 2. F1, F2 = full genome length primers 1 and 2, *radioactive probes. Abbreviations for gene names as in Figure 3.

5.7 LX-PCR and southern blotting to detect duplications

The existence of partially duplicated mtDNA molecules was studied using a PCR approach presented in Figure 5. Oligo Z (GGTAATCGCATAAAACCATCA, np 3247-3261 + 16072-16077 of human mtDNA), representing a sequence across the deletion breakpoint of one of the common sublimons, was used in combination with each of two different primers. These primers were X (GTTGGCCATGGGTATGTTGT, 3321-3302), located in the commonly deleted region and W (CCGGTCTGAACTCAGATCAC, 3080-3060), in the commonly undeleted region. A second approach using primers X and Y (TCCACCATTAGCACCCAAAG, 15976-15995), both located in the commonly deleted part of mtDNA genome, aimed at amplification of the wild-type 3.9 kb fragment plus any additional sequence from partially duplicated molecules, using LX-PCR conditions.

DNA from several tissues of three subjects found to contain various amounts of sublimons were southern blotted and hybridized as described in section 5.5.

5.8 Fluorescent PCR

To characterize specific subclasses of sublimons, semi-quantitative PCR analysis was carried out. Multiplex PCR reactions assaying the most prevalent 3.75 kb sublimon class used mtDNA primers mt3150 and mt16153FAM and primers DraIR and DraIFROX as an internal single-copy gene standard (Table 3, for the principle, see Figure 6). Primers mt8531 and mt2204 were used instead of mt3150 for the 7.4 kb deleted sublimon and for one of the ‘rare’ sublimons, respectively (Table 3). After a manual hot start for 3 min at 95 °C, a PCR cycle consisting of 30 sec at 95 °C, 20 sec at 59 °C and 20 sec at 72 °C was repeated 22 to 31 times and the fluorescent products were analysed by capillary electrophoresis using GeneScan software on an Applied Biosystems 310 Genetic Analyzer. An additional 7 min final extension step to maximize the 3’ A-overhang addition efficiency did not affect the relative or absolute amounts of the fluorescent products in any sample tested. Reaction products resolved at the nucleotide level were quantified as peak areas in the electrophoretogram and ratios computed of the amount of sublimon product versus the single-copy standard, at increasing cycle number. Data from points where saturation had not yet occurred were pooled, to extrapolate a mean copy number per cell, i.e. twice the number of single-copy equivalents, for all sublimons of the prevalent 3.75 kb class detected by the primers, considered collectively.

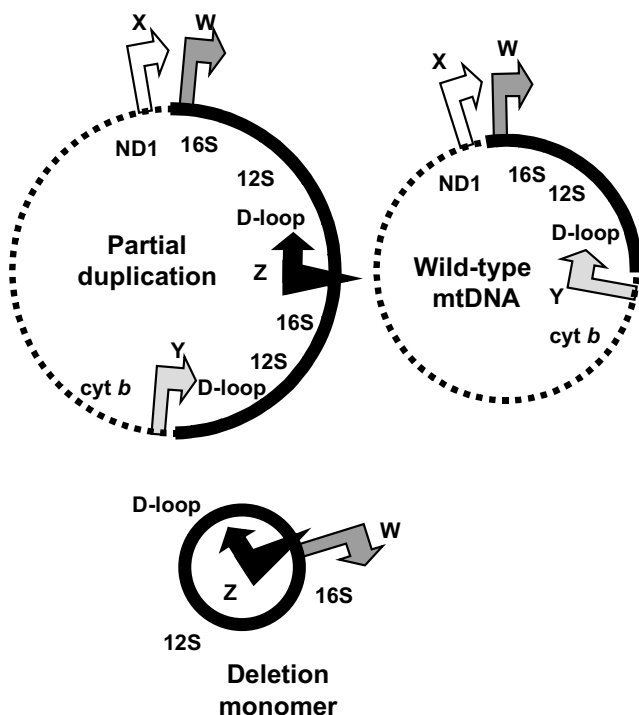


Figure 5. Principle of the analyses of the predicted partially duplicated mtDNA forms by long PCR. W, X, Y and Z = primers (see text), ND1 = NADH dehydrogenase 1 gene, 16S and 12S = 16S and 12S rRNA genes, cyt *b* = cytochrome *b* gene.

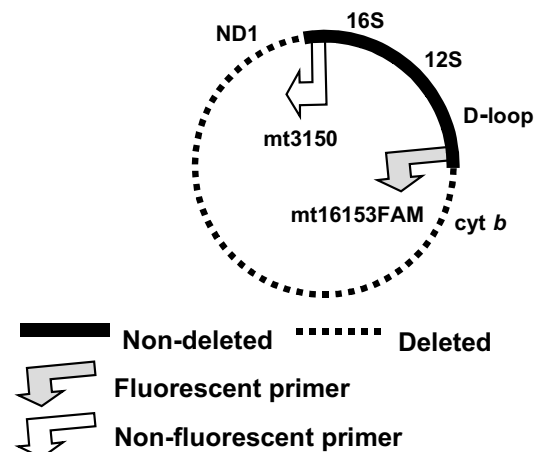


Figure 6. Detection of the common sublimon class by PCR with primers located across the sequence breakpoints. For abbreviations, see Fig. 5.

Table 3. Primers for fluorescent PCR analyses.

Primer	5' – 3' sequence	Co-ordinates*
mt3150	TACTTCACAAAGCGCCTTC	3150-3168
mt2204	TTCAAGCTCAACACCCACTA	2204-2223
mt8531	ACGAAAATCTGTTCGCTTCA	8531-8550
mt16153FAM	#CAGGTGGTCAAGTATTTATGG	16153-16133
DraIR	TCCCAAAGTGCCAGGATT	-
DraIFROX	**ATCATGGCTCATTGTAGCTTC	-

*np of human mtDNA, according to Anderson et al., 1981

incorporating a 5' FAM fluorescent label, **incorporating a 5' ROX fluorescent label.

6. Polymorphisms of the candidate genes

DNA extraction from frozen heart muscle in series B was carried as described in the previous section for mtDNA rearrangements. In series A, DNA was extracted from paraffin-embedded myocardial samples using QIAamp DNA mini kit (QIAGEN, Germany) according to the recommended protocol or by the method of Isola et al. (1994).

The polymorphisms studied are summarized in Table 4. Most of them are single-nucleotide polymorphisms (SNP) with two exceptions. In the ACE gene there is a polymorphic insertion/deletion of 300 bp, which was analyzed directly by PCR amplification and size fractionation of the products. Most of the SNPs destroyed or created a restriction endonuclease (RE) recognition site and were analyzed by PCR amplification and size fractionation of the products after incubation with the selected RE according to the manufacturer's instructions. The *AT1R* 1166A/C polymorphism was analyzed using solid-phase minisequencing method (Kainulainen et al., 1999). First, a 167 bp product containing the polymorphic site was amplified in a PCR reaction with primer 1 (AAGCTTTTGTTCAGAGCTTTAG, incorporating a 5' biotin label) and primer 2 (GTTCGAAACCTGTCCATAAAG). These PCR products were captured in streptavidin coated microtitration plate wells and made single stranded. A detection primer (CACTTCACTACCAAATGAGC) designed to anneal just before the polymorphic nucleotide enabled addition of a single ³H-labeled dATP or dCTP by DNA polymerase. The genotype of a given sample was defined by the ratio between the incorporated 3H-labeled nucleotides. Because of technical factors, the *CYP11B2*, *ADH3* and *CYP2E1* *DraI* –polymorphisms in the series A were analysed by modified PCR methods using novel oligonucleotide primers. The PCR reagents and REs used were supplied by the common manufacturers (Promega, Madison, WI, USA, MBI Fermentas, Vilnius, Lithuania, Finnzymes, Espoo, Finland).

Table 4. Polymorphisms of the candidate genes.

Gene	Type of polymorphism	Reference
<i>ACE</i>	I/D	Rigat et al., 1990
<i>AT1R</i>	1166A/C ¹	Kainulainen et al., 1999
<i>CYP11B2</i>	-344C/T	Kupari et al., 1998
<i>ADH2</i>	<i>MaeIII</i> ²	Groppi et al., 1990
<i>ADH3</i>	<i>SspI</i> ²	Groppi et al., 1990
<i>ALDH2</i>	<i>EcoRI</i> ²	Chao et al., 1994
<i>CYP 2E1</i>	<i>DraI</i>	Hirvonen et al., 1993
<i>CYP 2E1</i>	<i>MspI</i>	Uematsu et al., 1991
<i>CYP 2E1</i>	<i>PstI</i>	Hayashi et al., 1991b
<i>CYP 2E1</i>	<i>RsaI</i>	Hayashi et al., 1991b

¹Detection primer used:cacttcactaccaaatgagc, ²the common functional polymorphism

7. Statistical methods

Stepwise multiple linear regression analysis was used to study the relationship between postmortem cardiac measurements and the data on alcohol consumption and other potential independent variables (explanatory factors) (study I). The results of these analyses are given as standardized regression coefficients (beta). Stepwise ordinal polychotomous logistic regression analyses (study I) were carried out on cardiac measurements divided into four outcome classes (values 0-3) by the lower and upper quartiles and the median. The analysis is a generalization of the standard logistic model, but instead of two outcome classes there are four, which again have an ordinal scale. The results are summarized by odds ratios with 95 % confidence intervals, the interpretation of which is analogous to the standard model. In this analysis, when using categorical independent variables, they were classified into four classes, and the lowest class was always used as a reference. In the subgroup of men free of significant coronary artery disease at postmortem angiography, analysis of covariance (ANCOVA) was used to compare the cardiac measurements across the four different alcohol consumption groups. The factors besides alcohol consumption that were included in the multivariate analyses were age, body surface area, smoking (number of cigarettes/day) and the presence of hypertension and diabetes (both dummy coded variables). The findings at postmortem coronary angiography (number of vessels with >50% stenosis, 0-3) were used as a confounding factor in analyses of the total study group and to select the subgroup of men free of coronary artery disease for ANCOVA. For some analyzes (see appendix), sublimon abundance (copies/cell) was divided into three equally sized groups (N=98 in each) by the 33.33rd and 66.67th percentiles, corresponding to values 1.19 and 4.86 copies/cell,

respectively. Age was similarly divided into three equally sized groups of <46, 46-58 and >58 years (N=103, 100 and 97, respectively). The distribution of the confounding explanatory factors across the different alcohol consumption groups was studied using analysis of variance (ANOVA) and Pearson's chi squared test (studies I, II). Variables with grossly asymmetric distribution were square root or log-transformed before statistical analyses. The analyses were made using Statistica/Win (Version 5.0, StatSoft, USA) and SPSS/Win (Version 9.0, USA) on personal computer or the BMDP Statistical Software (Version 1990, USA) on a SUN/UNIX mainframe.

RESULTS

1. Characteristics of the autopsy series (I, II)

The autopsy series is characterized in Table 5. The median daily alcohol consumption in the series was high, 60.5 g, and 46 % of the subjects with reliable drinking history and daily alcohol dose were classified as heavy or very heavy drinkers (>72 g/day). Smoking was frequent, 82 % of all men were smokers (proportions of current and ex-smokers were 69 % and 13 %, respectively). Significant coronary narrowings (presenting >50 % of the lumen diameter) were found in 38.8 % of the subjects.

The alcohol consumption groups are characterized in Table 6. The prevalence of significant coronary artery disease decreases substantially from light drinkers (68 %) to moderate (50 %), heavy (37 %) and very heavy drinkers (20 %). The mean age of the men decreased and the amount of cigarettes smoked increased from the lowest to the highest consumption group, while hypertension and diabetes were equally distributed to the drinking categories (Table 6).

Table 5. Characteristics of the men in the autopsy series

	Valid N	Mean (SD) or frequency	Median	Range
Age (years)	700	53.1 (9.6)	54.0	33 - 70
Height (cm)*	699	175 (7.1)	175	153 - 195
Weight (kg)*	699	75.3 (16.5)	73	38 - 145
Body surface area (m ²) [†]	699	1.90 (0.21)	1.89	1.34 - 2.73
Alcohol consumption (g/day)	435	95.4 (101.7)	60.5	0.0 - 510
Smoking (cigarettes/day)	500	17.3 (13.9)	20	0 - 50
Hypertension#	477	107 (22.4 %)		
Diabetes#	477	113 (23.7 %)		
Coronary artery disease at autopsy [‡]	641			
Not found		399 (62.2 %)		
1 vessel disease		109 (17.0 %)		
2 vessel disease		71 (11.1 %)		
3 vessel disease		62 (9.7 %)		
Myocardial infarction at autopsy	700			
Any		183 (26.1 %)		
Old		149 (21.3 %)		
Recent		85 (12.1 %)		

SD = standard deviation. * Measured at autopsy. [†] Calculated from height and weight (Dubois and Dubois, 1916). #As reported by the interview. [‡] Number of arteries with > 50 % stenosis at postmortem angiography.

Table 6. Characteristics of the men with reliable drinking history by categories of daily alcohol consumption

	Average alcohol consumption (g/day)				P value
	<12 (N=109)	12-72 (N=105)	72-180 (N=103)	>180 (N=76)	
Age	56.4 (9.9)	53.7 (9.1)	52.7 (9.3)	48.8 (8.7)	<.00001
Body surface area (m ²)	1.95 (.22)	1.92 (.19)	1.87 (.19)	1.91 (.23)	.058*
Hypertension#	27/109	25/105	19/103	15/76	.64
Diabetes#	26/109	29/105	21/103	20/76	.64
Coronary artery disease [†]	75/109	54/105	39/103	15/76	<.0001
Smoking (cigarettes/day)	13.5 (13.0)	17.9 (15.2)	18.2 (13.3)	19.2 (14.1)	.094*

Data are mean (SD) or frequencies. *P* values are for differences across alcohol consumption groups from ANOVA and Pearson's chi squared test. *For log transformed data. # As reported by the interview. [†] > 50 % coronary luminal stenosis at postmortem angiography.

2. Autopsy diagnoses of alcohol-related heart muscle disease (unpublished results)

In the series B, there were 13 cases with a diagnosis alcoholic cardiomyopathy as the cause of death (ICD-9 classification: Cardiomyopathia alcoholica 4255A). Seven additional cases had a diagnosis of alcoholic cardiomyopathy, which was not the cause of death. The diagnostic classification used at the time of the series A collection (ICD-8) did not include alcoholic cardiomyopathy, instead there were two cardiomyopathy diagnoses, cardiomyopathia alia definita (425,08) and cardiomyopathia NUD (425,09). We included as alcoholic cardiomyopathy six cases of diagnosed cardiomyopathy from the series A, among which retrospectively judged alcohol was the only detectable factor to cause myocardial disease. Thus the prevalence of alcoholic cardiomyopathy was in the whole series 26/700 (3.7 %).

To estimate the presence of heavy alcohol use (> 80 grams/day) or alcoholism in cases where the daily alcohol dose could not be calculated, the questionnaire graded the men as alcoholic or non-alcoholic in 602 cases out of the total 700. Based on this data, the prevalence of any alcohol-related heart muscle disease was retrospectively estimated (Table 7). The findings at autopsy, which were included were dilatation, hypertrophy, fibrosis, adipose infiltration and unknown myocardial disease. Findings in cases who in addition to alcohol use had any other potential cause of myocardial disease, mainly significant coronary artery disease or myocardial infarction, diabetes or aortic stenosis, were not classified as alcohol-related. Among the 320 autopsied cases with history of alcoholism or heavy alcohol use, 42 (13.1 %) were retrospectively found to have an alcohol-related heart muscle disease diagnosis (Table 7).

Table 7. Any diagnosed alcohol-related heart muscle disease among cases with moderate and heavy alcohol consumption. N=602.

Alcohol consumption*	Alcohol-related heart muscle disease	
	None	Yes
Moderate	279 (98.9 %)	3 [#] (1.1 %)
Heavy	278 (86.9 %)	42 (13.1 %)

*Estimate based on history of alcohol use in cases, where the exact daily alcohol dose could not be calculated. **Known history of hypertension. #These cases were diagnosed as alcohol-related by the pathologist, although the later interview data did not support heavy use of alcohol.

3. Associations of cardiac measurements with explanatory factors other than the daily alcohol dose (I)

The associations of explanatory factors with cardiac measurements were first analyzed among all men with complete covariate data by stepwise multiple regression analysis. Because the effects of some of the factors did not seem to be simply linear, polychotomous logistic regression analysis was performed on the same variables.

Of the studied independent factors (age, body size, severity of coronary artery disease, cigarette consumption, history of hypertension, history of diabetes, alcohol consumption), body size was the major determinant of all measured cardiac dimensions except for wall thicknesses. Age, severity of coronary artery disease and history of hypertension each had influence on LV weight and at least one other LV measurement. The severity of coronary artery disease had a direct relationship to the LV cavity area, while LV wall thickness was determined more by the history of hypertension. Smoking had a small effect on RV cavity area, but not on any other variable. History of diabetes did not influence any of the measured variables in the stepwise analyses. These associations were confirmed by the polychotomous logistic regression analysis.

4. The effects of daily alcohol dose on cardiac measurements among all men (I)

Using stepwise multiple regression analysis, alcohol consumption (square root of daily alcohol dose) was found to independently influence total heart weight ($\beta=0.17$, $P<0.001$) and RV cavity area ($\beta=0.14$, $P=0.007$). Both of these variables increased with increasing daily alcohol intake. The non-linear polychotomous logistic regression analysis confirmed these associations and revealed an additional effect of alcohol on LV weight. This relationship was neither simple nor linear, both moderate (12-72 g/day) and very heavy (> 180 g/day) consumption having a positive effect, while daily doses of 72-180 grams had only a minor effect. Among all men, the daily alcohol dose did not significantly influence LV cavity area.

5. The effects of daily alcohol dose on cardiac measurements among men free of coronary artery disease and myocardial infarction at autopsy (I)

To analyze further the effects of alcohol on heart anatomy, a subset of men without significant coronary artery disease (< 50 % luminal narrowings at autopsy) and myocardial infarction were separated from the rest. Alcohol consumption was classified according to the daily alcohol dose into groups of < 12 , 12-72, 72-180 and > 180 g/day. In ANCOVA, adjusting for age, (log) body surface area, smoking (log number of cigarettes/day), history of diabetes and history of hypertension, daily alcohol dose had a significant effect on total heart

weight, LV cavity area and posterior wall/cavity area –ratio and RV cavity area (Table 8). LV cavity area decreased with increasing alcohol intake up to 180 g/day ($P=0.054$ for the overall effect, Scheffé’s post hoc test $P=0.007$ for < 12 g/day vs. 72-180 g/day) (Figure 7), after which the area increased. RV cavity area showed a statistically non-significant trend towards decrease from light to heavy consumption and an increase from dose 180 g/day on ($P=0.005$ for the overall effect, Scheffé’s post hoc test $P=0.005$ for 12-72 g/day vs. >180 g/day) (Figure 7). The LV posterior wall/cavity area –ratio showed a pattern, which was essentially a mirror image of the LV cavity changes, with an increase up to 180 g/day and a decrease after that ($P=0.036$ for the overall effect, Scheffé’s post hoc test $P=0.003$ for < 12 g/day vs. 72-180 g/day). Daily alcohol intake had in addition a statistically significant effect on total heart weight ($P=0.015$) and a nearly statistically significant effect on LV weight ($P=0.074$).

All analyses were repeated without history of hypertension as a covariate, assuring that any possible hypertension-mediated effects of alcohol could be accounted for. The statistical associations remained essentially unchanged.

Table 8. Cardiac measurements in relation to daily alcohol consumption in men free of coronary artery disease and myocardial infarction at autopsy

Measurement	Daily alcohol consumption				<i>P</i> *
	<12 g n=31-34	12-72 g n=44-50	72-180 g n=56-59	>180 g n=57	
Heart weight (g)	442 (16)	461 (14)	440 (10)	466 (15)	.015
Left ventricular					
Weight (g)†	193 (7.3)	207 (8.4)	190 (5.2)	204 (8.7)	.074
Cavity area (cm ²)	11.0 (1.0)	9.4 (1.0)	7.7 (0.7)	10.0 (0.9)	.054
Anterior wall (mm)	16 (0.4)	17 (0.5)	17 (0.4)	17 (0.4)	.10
Posterior wall (mm)	16 (0.5)	17 (0.6)	17 (0.4)	17 (0.4)	.47
Posterior wall/cavity area –ratio (mm/cm ²)	2.0 (0.3)	3.1 (0.5)	5.2 (0.9)	3.7 (0.8)	.036
Right ventricular					
Weight (g)†	65 (3.3)	67 (2.3)	69 (3.2)	66 (3.4)	.45
Cavity area (cm ²)	9.8 (0.8)	8.9 (0.7)	8.6 (0.6)	10.8 (0.7)	.005

The data are mean (SEM, standard error of mean). **p* value from ANCOVA with log body surface area, age, smoking (log number of cigarettes/day), history of diabetes and history of hypertension as covariates.

†Number of cases was 21, 34-35, 40 and 36.

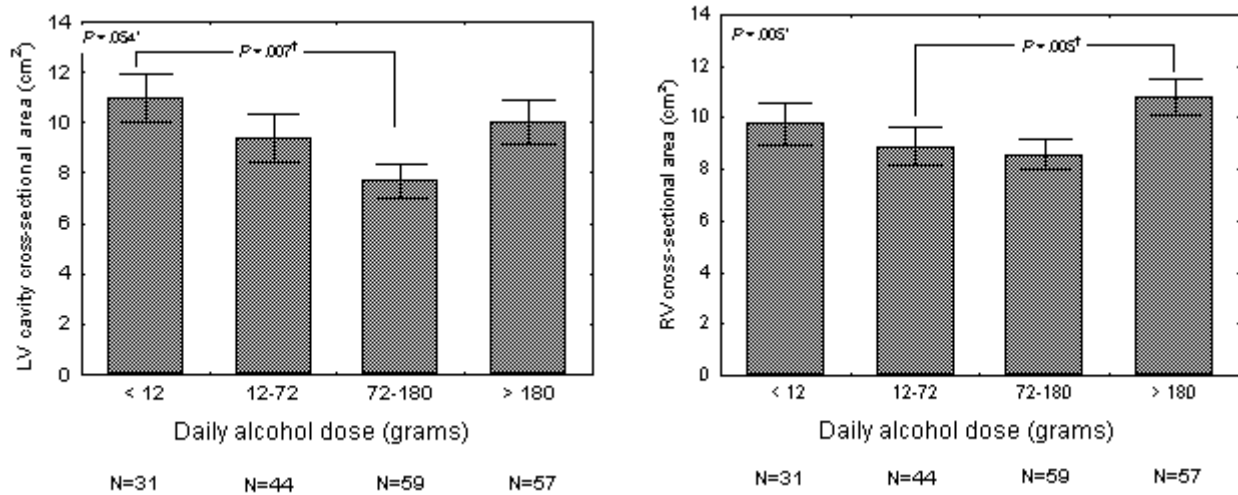


Figure 7. Left and right ventricular (LV and RV) cavity sizes in relation to daily alcohol dose in men free of coronary artery disease.

6. Interactions of alcohol and coronary artery disease on the LV measurements (II)

To characterize further the modifying effects of variable degrees of coronary artery disease on the development of the alcohol-related anatomical changes, the men were divided into three roughly equally sized groups according to the most severe coronary stenosis (< 30 %, 30-60 % and > 60 %, Table 9). In 2-way ANCOVA adjusting for age, body size, smoking, history of diabetes and history of hypertension, CAD severity had a statistically significant effect on LV cavity area ($P=0.030$ for overall effect) and posterior wall/cavity area –ratio ($P=0.014$ for overall effect), but not on wall thicknesses or LV weight. In these 2-way analyses, the overall effect of alcohol dose was not statistically significant on any of the LV measurements.

There were statistically significant interactions between the effects of daily alcohol dose and CAD severity on LV posterior wall/cavity area –ratio ($P=0.018$ for interaction) and LV cavity area ($P=0.037$ for interaction). This highly suggested that the effects of the alcohol dose are influenced by the degree of concomitant coronary artery stenosis. In the further subgroup analyses it was shown that in men with no or only mild CAD (< 30 % maximal coronary artery stenosis), LV posterior wall/cavity area –ratio increased with increasing daily alcohol intake up to 180 g/day ($P=0.012$). The men drinking heavily (72-180 g/day) had increased ratios compared with light drinkers (< 12 g/day) ($P=0.021$ from Scheffé's post hoc test), which seemed to reflect mainly smaller LV cavity areas among these men ($P=0.021$ from Scheffé's post hoc test <12 g/day vs. 72-180 g/day). The changes were directionally similar, but smaller and statistically non-significant among men with 30-60 % maximal coronary artery stenosis. In contrast to the rest of the men, the group with severe coronary artery narrowings (> 60 % maximal diameter stenosis) showed opposite LV changes. Increasing alcohol doses among them were associated with a trend towards a larger LV cavity area and smaller LV posterior wall/cavity area –ratio.

We classified CAD alternatively by the number of affected vessels (having > 50 % luminal stenosis, coded from 0 to 3). The results were directionally similar to those obtained by the present classification, but did not reach statistical significance (P=0.10-0.15). One reason may have been that in the series heavy drinking was seldom combined with multivessel CAD, which reduced the size of some of the subgroups in 2-way ANCOVA too much (data not shown).

Table 9. Subgrouping of the men in the four alcohol groups by the most severe coronary stenosis

	<12 g N=109	12-72 g N=105	72-180 g N=103	>180 g N=76	<i>P</i> value
Most severe stenosis					<.00001
<30%	15/109	25/105	36/103	29/76	
30-60%	34/109	41/105	39/103	40/76	
>60%	60/109	39/105	28/103	7/76	

P value is from Pearson's chi squared test.

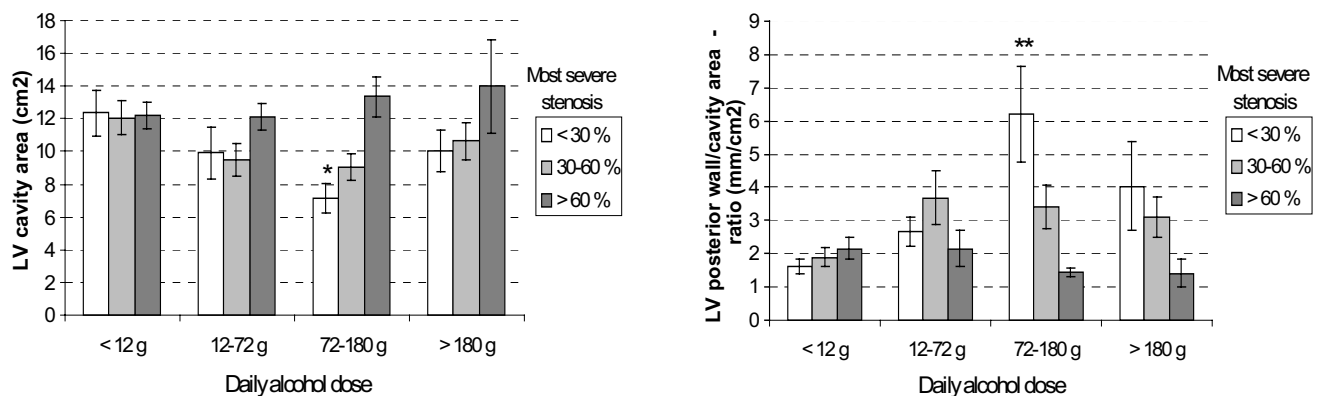


Figure 8. Left ventricular (LV) cavity area and posterior wall/cavity area –ratio in relation to daily alcohol consumption and maximal coronary stenosis. *<12 g/day vs. 72-180 g/day, *P*=.021, Scheffé's *post hoc* test. **<12 g/day vs. 72-180 g/day, *P*=.021, Scheffé's *post hoc* test.

7. mtDNA rearrangements in the heart (III, IV, V)

The whole mtDNA genome was successfully amplified with two different sets of adjacent, outwardly oriented primers, sets 1 and 2 (OK1H/OK2L, nt 161-160 of human mtDNA and FR6H/OK6L, nt 3013-3012, respectively) (Figure 3, 4). In principle, all rearranged mtDNA molecules can be detected in one PCR reaction as products differing in size from the wild-type, provided they still contain the priming sites. A product corresponding to the full-length 16.6 kb linear mtDNA was detected in all cases. Various shorter products were also detected in every case, some of them identical and some differing, mostly quantitatively, between the samples. Both primer pairs yielded a prominent product of about 4 kb, in addition multiple bands sized 5-12 kb were seen, some of them not properly separated in the gel (Figure 9). Extra long agarose gel electrophoresis revealed at least 20-30 visible LX-PCR products. Most, although not all of the products generated by the two primer pairs were similar, suggesting mispriming artefacts to be uncommon. Products shorter than ca. 3 kb were usually detected only by either one of the full-genome primer sets, which most probably derives from the fact that the two primer sets are located 3 kb apart on the mtDNA sequence, and no product shorter than that can include both of the priming sites unless it contains multiple deleted regions. The LX-PCR products were blotted into a nylon membrane and southern hybridization was carried out using two different probes, one for the junction region of 16S rRNA and ND1 genes and one for the junction region of ND4 and ND5. The genome-length wild-type LX-PCR product and most of the shorter ones detected on agarose gel gave strong hybridization signals with the 16S/ND1 probe, but no single band except for wild-type was detected using the ND4/ND5 probe. In general, neither of the probes hybridized to products < 2 kb in length, which is also logical as the distance between the primer pair 1 and the 16S/ND1 probe is about 2.8 kb. One possible exception was a ca. 1 kb product, which to be a *bona fide* deleted mtDNA, would have to bear at least two different rearrangements, removing over 15 kb of the sequence. However, the two probes may have detected entirely different products of the same size.

Several different 'sub-genomic' primer pairs were used to generate overlapping products having predicted sizes of 4.8-13.7 kb from the wild-type mtDNA (Figure 3, 4, Table 1, 2). The predicted full-length products were detected in every case, plus several shorter products specific to each primer pair. The shorter products representing putative rearranged mtDNA molecules were not produced equally by the primer sets. For example, the mostly overlapping primer sets 7 (FR6H/OK2L, nt 3013-160 in human mtDNA) and 6 (OK1H/FR44L, nt 161-12553) seemed to generate discordant products, set 7 giving abundant shorter products hybridizing to the 16S/ND1 probe, while set 6 gave none. This finding however can be explained by later sequence analyses, which showed that set 7 primes on a region of mtDNA most commonly preserved among rearranged molecules, while no rearrangements, which would have preserved the priming site of FR44L (nt12553-12572), have been detected. The other primer sets giving potentially controversial results were set 2 (OK1H/FR32L, nt 161-7840), which gave almost no non-wild-type products and set 4 (FR6H/FR44L, nt 3013-12553), which gave 4-5 quite prominent shorter products. According

to the later sequence analyses, no deletion break-points were found between the primers of set 2, in line with the lack of LX-PCR products. The products from primer set 4 most probably represent rearranged molecules involving a small region, which was not covered in the later cloning and sequencing work. Naturally they might also be mispriming artefacts or products from nuclear pseudogenes.

7.1 LX-PCR of ACM and controls

DNA extracted from left ventricular myocardium of autopsied alcoholic cardiomyopathy (ACM) cases (N=8) yielded LX-PCR products, which did not differ from control hearts (N=10) in a systematic manner, either qualitatively or quantitatively (Figure 9). This suggests that the amount of large mtDNA rearrangements is not increased in ACM hearts and that the spectrum of various putative deleted mtDNA species is not different from controls.

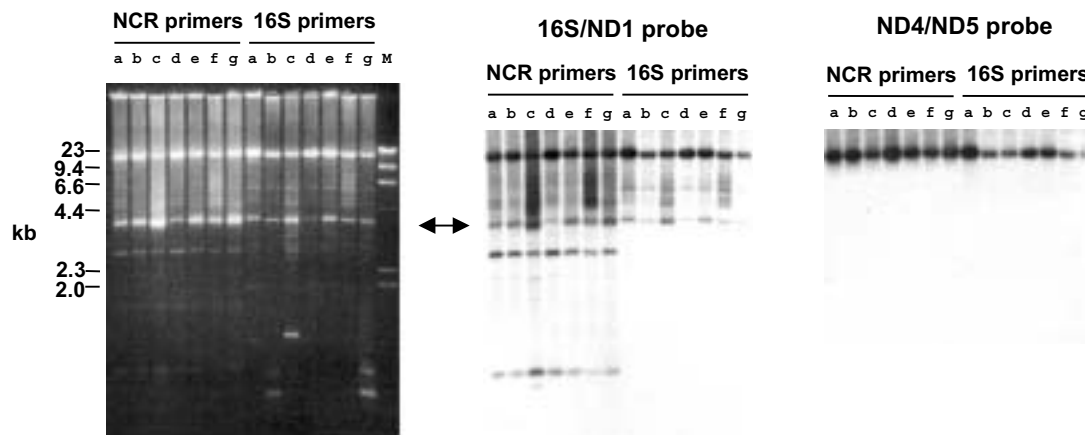


Figure 9. LX-PCR products on agarose gel and southern blots with two probes (16S/ND1 and ND4/ND5). a, b, c = controls, d, e, f, g = ACM cases, M = λ /HindIII marker, sizes of fragments in the other side of the gel. Pri = primers. All samples show a strong band of 16.6 kb corresponding the full-length wild-type mtDNA. The arrow denotes a prominent PCR product of ca. 4 kb.

7.2 Various artefacts arise from the LX-PCR methodology

Products of differing lengths are known to be synthesized with variable efficiencies by LX-PCR. Because the amount of the shorter vs. full-length wild-type products seemed to crudely correlate with the length of the amplicon, LX-PCR was applied on a wide dilution series of template DNAs of different samples. At very high template concentrations (ca. 0.1-1 μ g/reaction), the shorter, putative deleted mtDNA molecules were synthesized efficiently, while the full genome-length product was only faint or even absent. As the template concentration in the PCR reaction mix was gradually reduced, the full-length product

appeared in increasing amounts. Some concentrated samples showed at first almost exclusively ‘deleted’ LX-PCR products. Strikingly, after being diluted to give the optimal full genome-length amplification, the same samples gave hardly any other products than the full-length 16.6 kb band. As an example, one of the control heart samples gave abundant shorter LX-PCR products at high template concentrations (Figure 10). After dilution of the DNA template to enhance the amplification of the full genome-length product, the latter clearly dominated over the ‘deleted’ molecules, despite the preference for the amplification of shorter products by LX-PCR. A southern blot of *Bam*HI-digested genomic DNA of the same heart sample, probed for the 16S/ND1 region, did not easily detect any signal except for the linearized wild-type mtDNA (*Bam*HI cuts once at nt 14258 of human mtDNA).

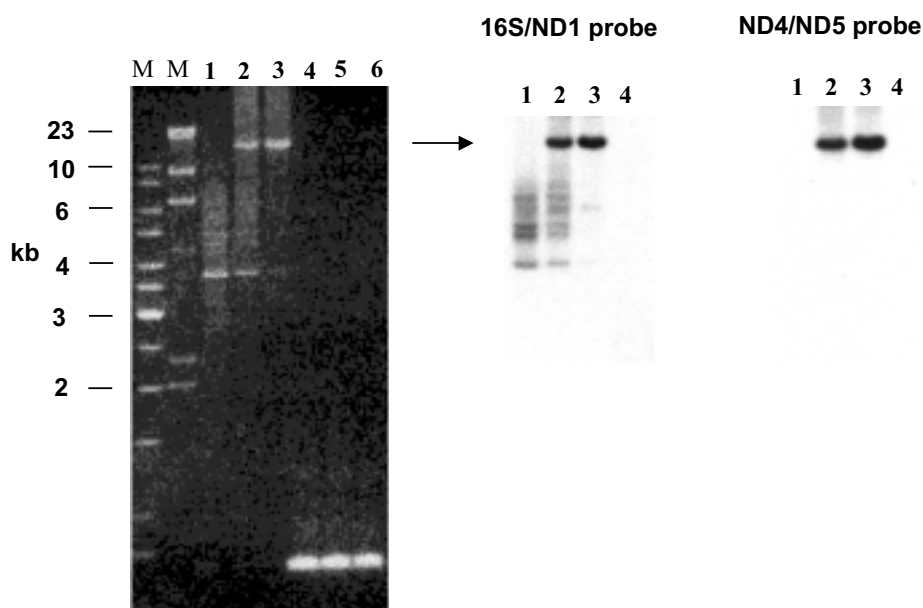


Figure 10. Effect of dilution of template DNA prior to LX-PCR analysis, agarose gel and southern blot probed separately by 16S/ND1 and ND4/ND5 probes. Arrow denotes the 16.6 kb full genome-length product. Template DNA included in a 50 μ l reaction was as follows: lane 1 - 150 ng, lane 2 - 30 ng, lane 3 - 6 ng, lane 4 - 1.2 ng, lane 5 - 0.25 ng, lane 6 - 0.05 ng. M = 1 kb and λ /*Hind*III markers

To test further the sensitivity of LX-PCR, DNA samples with known fractions of previously mapped mtDNA deletions from KSS (Kearns-Sayre syndrome) and ad-PEO (autosomal dominant progressive external opthalmoplegia) patients were used. The LX-PCR mix containing a fixed concentration of control cell-line (143B osteosarcoma cybrid) DNA was spiked with known amounts of the deleted patient’s DNA. It was found that the deleted molecules could be detected by regular EtBr staining when representing only ca. 0.2 % of all mtDNA molecules in the LX-PCR reaction. By southern blotting and hybridization to a

radioactive probe, the detection limit was lowered a further five-fold, which means that aberrant molecules of this kind can be detected when present in one copy per cell or even less (a cell contains on average 1000-5000 copies of the mitochondrial genome). Unlike the LX-PCR products deriving from putative deletions in the control myocardium, both of the pathological deletions remained the most prominent products, even at the lowest dilutions of the samples.

7.3 Characterization of mtDNA rearrangements in the heart

The LX-PCR products from the heart of one of the controls (male, 49 years) were further characterized by plasmid cloning and automated DNA sequencing. First the mixture of the various full genome-length LX-PCR products was cloned at random, later preselecting for products > 4 kb. This was done because both PCR and plasmid cloning prefer shorter fragments. The maximal size of effectively clonable fragments was around 6-7 kb, but some of the detected LX-PCR products, although present in low amounts, exceeded this length. Thus sub-genomic LX-PCR was used to obtain products from rearranged molecules, whose sequence break points were possibly further down the mtDNA sequence. At the same time we aimed at rearrangements preserving the light strand origin of replication (O_L), so the other primer used was designed to locate about 500 bp before O_L . Single bacterial colonies obtained were screened by PCR, and because of some prominent products, e.g. one around 4 kb, the clones were not picked completely randomly, but instead to cover inserts of all sizes. 65 clones (42 derived from full genome-length and 23 from sub-genomic LX-PCR) were sequenced (Table 10); shorter inserts in their entire length, longer ones until a sequence break-point corresponding to the size of the assumed rearrangement was encountered. Only one of the 65 clones turned out to a mispriming artefact generated during PCR, all other sequenced clones represented correctly primed products containing a mtDNA rearrangement in the middle of the sequence.

The rearrangement break-points were found to locate preferentially in certain regions of the mtDNA genome, highly suggesting non-random distribution. The most prominent hotspot region was at the end of the D-loop (around nt 16070), which is designated as the common break-point region (CBR). This was involved in over 80 % (52/64) of the rearrangements sequenced. The other break-points (12/64) in this region of the genome were found at random positions further along the mtDNA sequence, the most distant at nt 15405. On the other side of the mtDNA genome several less prominent hotspots regions were detected. One of these was around nt 3260 (8/64), and the sequence of these clones continued from around 16070 (6/8) or nt 16034 (2/8). These 6 clones represent the highly prominent LX-PCR product of 3.75 kb, termed later as the 'common sublimon'. A second commonly seen rearrangement, corresponding to a 7.4 kb deletion, was bounded on one side by the CBR and on the other by nt 8637-8648, an exact 12 bp repeat of the CBR sequence.

Aside from the large-scale rearrangements characterized, the sequence data collected showed very few aberrations from the reference mtDNA sequence. Most of these could be attributed either as PCR-induced mutations or polymorphisms. Characteristic features of

nuclear pseudogenes, namely the widespread scatter of low level substitutions and micro-deletions, was not detected. Less than half (20/56) of the unique sublimon break-points included direct repeats of > 1 bp in length, and most of these were very short, only eight being of 6 bp or more. One clone had a 1 bp insertion at the break-point.

Table 10. Sublimons represented as cloned PCR products from control heart muscle

Clone	Size (bp)	Direct repeat (bp)	1st break-point (np)	2nd break-point (np)	Note
6	940	6	633-639	16262-16268	
11	1351	11	887-898	16105-16116	
2	2170	0	1672	16071	
8	2267/8	1	1770/1771	16071/16072	
16	2396/7	1	1906/1907	16078/16079	
10	2402/3	0	1906	16072	
5, 15	2562/3	4	2064-2068	16070-16074	
3, 13	2826/7	0	2333	16075	
17	2829/30	1	2333/2334	16072/16073	
4	3061/2	0	2540	16047	
18	3278/9	0	2781	16071	
14	3308/9	0	2818	16078	
9	3753	1	3255/3256	16071/16072	
21	3754	0	3255	16070	
1	3759	1	3262/3263	16072/16073	
19, 22	3759/60	2	3261-3263	16070-16072	
23	3761/2	1	3262/3263	16069/16070	
20	3804/5	0	3270	16034	(1)
24	3806/7	0	3272	16034	
5B	3986/7	0	3485	16067	(2)
4B	4045/6	1	3548/3549	16071/16072	
2B	4137/8	3	3308-3311	15738-15741	
1B	4175/6	0	3575	15968	
3B	4192/3	0	3692	16068	
48	4236/7	6	3736-3742	16068-16074	
43	4323/4	0	3826	16071	
38	4599	10	3568-3578	15537-15547	(3)
37	4636/7	2	4139-4141	16071-16073	
33	4677/8	1	3548/3549	15439/15440	
34, 36	4866/7	0	4376	16078	
40	4952/3	4	4454-4458	16070-16074	
45	4979/80	4	4482-4486	16071-16075	
47	5692/3	0	5195	16071	
42	5820/1	0	5328	16076	
49	5821/2	2	5328-5330	16075-16077	
44	6168/9	0	5671	16071	
35	6284/5	1	5786/5787	16071/16072	(4)
71	7759/60	0	7268	16077	
92	8125/6	1	7628/7629	16071/16072	
72	8137/8	7	7508-7515	15939-15946	

90	8141/2	0	7644	16071	
80	8163	1	7666/7667	16071/16072	
70	8228/9	0	7732	16072	
81	8308/9	4	7807-7811	16067-16071	
69	8315/6	6	7818-7824	16071-16077	
89	8417/8	2	7919-7921	16070-16072	
73	8526/7	5	8029-8034	16071-16076	
79	8530/1	2	8031-8033	16069-16071	
88	8562/3	0	7399	15405	(5)
74	8590/1	0	8093	16071	
91	8826/7	1	8291/8292	16033/16034	
87	8874/5	1	8372/8373	16066/16067	
82	9060/1	3	8561-8564	16069-16072	
66-68, 83-85	9131/2	12	8636-8648	16073-16085	
65	9131/2	12	8636-8648	16073-16085	(6)
7	n/a	n/a	n/a		(7)

Notes:

- (1) t → c (np 3264) near break-point
- (2) one base-pair insertion (t) at break-point
- (3) additional 8 bp direct repeat on opposite sides of break-point
- (4) a → g (np 16074) near break-point
- (5) 7 bp direct repeat adjacent to break-point
- (6) t → a (np 8634) near break-point
- (7) mis-priming artefact at np 16257

The junctional oligo Z, designed to extend only 6 bp across the re-arrangement break-point of clones 19 and 22, gave in combination with primer X a product of 3.8 kb, which was the predicted size of a partially duplicated molecule (Figure 11). In another PCR reaction, the primer Z gave with primer W a product of 3.6 kb, which could be derived from either a deletion multimer or a partial duplication. The identity of these PCR products was confirmed by direct sequencing of the gel-purified fragments. A heart DNA sample, which was not found to contain detectable amounts of sublimons, did not give either one of these sublimon-specific products. Primers X and Y, which lie in the wild-type mtDNA just outside the common sublimon sequence in a predicted partially duplicated molecule, gave PCR products of 3.8, 7.6 kb and ~11.5 kb. The most intense product 3.8 kb would be predicted to derive from wild-type mtDNA and the 7.6 and 11.5 kb products match exactly the prediction for a partial duplication and triplication, respectively.

A sublimon junctional oligo probe was used to probe various digests of heart and muscle DNA. In addition to signal from the wild-type mtDNA, weak signal from three shorter bands was seen in *Bam*HI digest (Figure 12). These matched exactly the sizes of predicted circular mono-, di- and trimers of the prevalent 3.75 kb sublimon sequence (Figure 12). Other digests (not shown) with enzymes cutting inside the prevalent 3.75 kb sublimon showed hybridization signal to bands of the linear size predicted for the rearrangement monomers (3.75 kb).

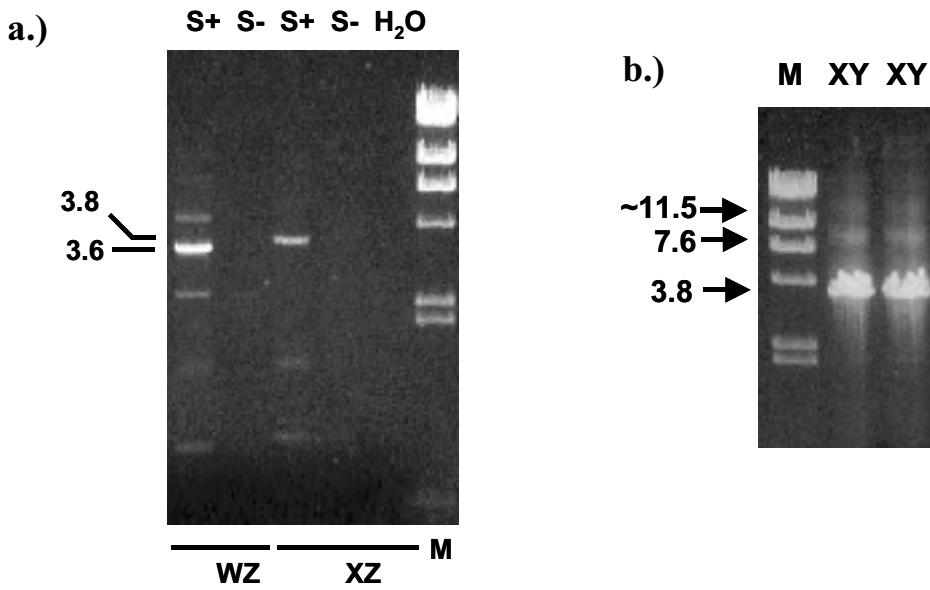


Figure 11. (a) Detection of partially duplicated mtDNA molecules in control heart samples, which either contain (S+) or lack (S-) sublimons. H₂O = water control. (b) LX-PCR amplification of the predicted partially duplicated and triplicated mtDNA molecules in control adult heart samples. M = λ *Hind*III digest. WZ, XZ and XY = primer pairs used, see methods.

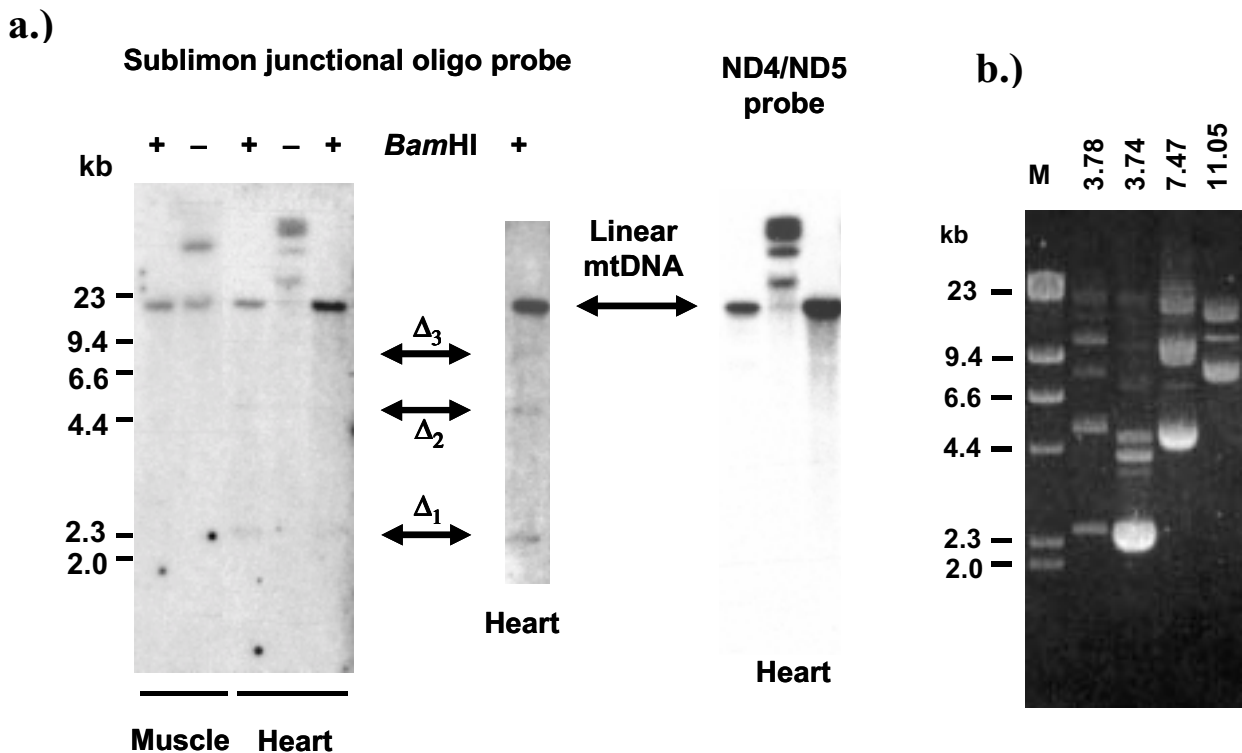


Figure 12. (a) Detection of sublimons by southern blotting with a junctional oligo probe. After stripping, the same filter was hybridized to a ND4/ND5 –probe. In addition to signal from linear wild-type mtDNA, shorter bands (Δ_{1-3}) are seen, matching exactly the predicted size of circular deletion mono-, di- and trimers, as presented in (b) by supercoiled circular plasmids of different sizes (3.78, 3.74, 7.47 and 11.05 kb).

8. The prevalent class of sublimons in heart of a series of 300 autopsied males (unpublished data, see appendix)

The semi-quantitative PCR analyses (see Methods) were successful in 294 cases of the total 300 males collected during years 1991-92. DNA samples analyzed originated from left ventricular muscle of the heart, frozen immediately after sampling (see Methods). The ca. 20 different molecular species belonging to the most prevalent (3.75 kb) class of sublimons were collectively present in myocardium at 0-90.6 copies per cell (mean 6.1, SD 9.9). In 28 cases out of the 294 the amount of sublimons was under the detection limit of ca. 0.1 copies/cell. Mean ages for the three sublimon groups (N=98 in each), defined by the 33.33rd and 66.67th percentiles, were 48.5, 51.6 and 56.6 years.

Complete covariate data was available for 101 cases. In multiple regression analysis (Table 11), using 'enter' -mode to force all the independent variables into the model, the number of sublimon copies per cell was determined statistically significantly by age ($\beta=0.43$, $P<0.0001$) and the number of cigarettes smoked ($\beta=0.25$, $P=0.012$). Forward and backward stepwise analysis methods gave the same significant factors. As was evident from the scatterplot with age (Figure 13), there was no clear threshold value of age for sublimon accumulation, and high numbers were seen among subjects of all ages.

Table 11. Multiple regression analysis results of potential factors affecting sublimon abundance, given as standardized regression coefficients (beta) and *P* values. N=101.

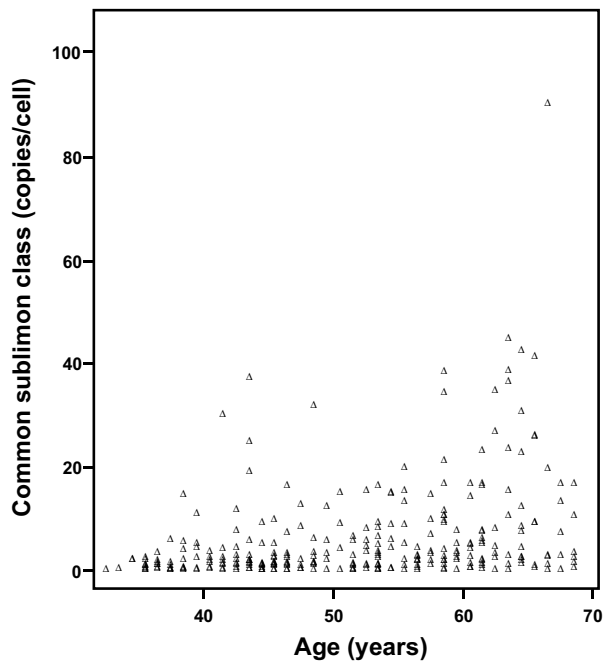
Model	Beta	P value
Age	0.43	<0.0001
Smoking intensity (cigarettes/day)	0.25	0.012
Alcohol consumption (grams/day)	-0.14	0.14
BSA	0.12	0.21
<i>Post mortem</i> time	0.09	0.33
CAD*	-0.02	0.85
Diabetes†	-0.001	0.95

*maximal luminal stenosis of any coronary artery

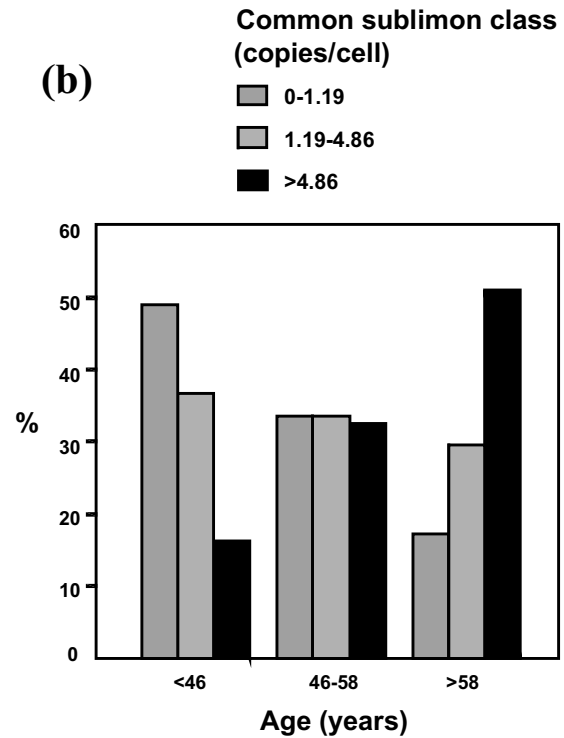
†as recorded by the interview

BSA = body surface area, CAD = coronary artery disease

(a)



(b)



(c)

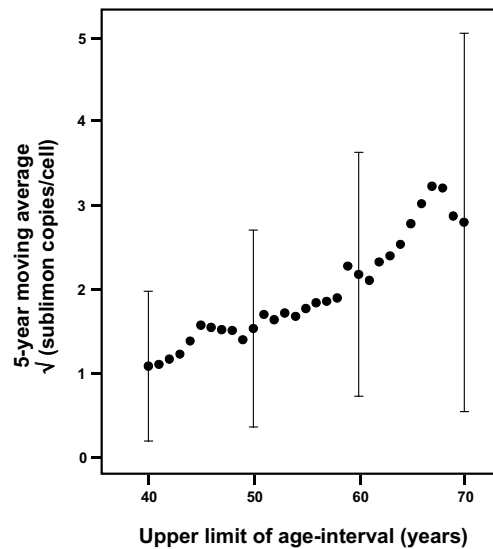


Figure 13. Abundance of the common sublimon class vs. age, $N=294$. (a) Sublimon copy number plotted against age. (b) The distribution of the men according to age and sublimon copy number, both variables divided into three equal sized groups by 33.3th and 66.7th percentiles. Cases in each age group sum up to 100%. (c) The same data after square-root transformation and grouping into 5-year age intervals, plotted as a moving average (mean square-root of sublimon abundance, expressed as copies per cell, for all individuals within each 5-year age interval). Standard deviations are shown only for the intervals ending at ages 40, 50, 60 and 70, but are similar for all intermediate intervals.

In ANCOVA, adjusting for age, the severity of coronary artery disease (as the number of vessels having > 50 % luminal stenosis) was not associated with sublimon levels ($P=0.89$). Either the existence of myocardial fibrosis ($P=0.53$) or an autopsy diagnosis of dilatation, hypertrophy or other myocardial pathologic finding (cardiomyopathy, fatty infiltration of myocardium) ($P=0.62$), had no effect on sublimon copy number. Whether the subject was a smoker (including previous smokers) or a non-smoker, or had a history of diabetes or not, was not related to the sublimon abundance ($P=0.54$ and $P=0.24$, respectively). In ANCOVA, none of the cardiac dimensions, adjusted for body surface area (BSA) and age, was related to sublimon copy number (divided into three groups, see Methods) (data not shown). When all of the analyses were repeated with age as an independent factor (divided into three groups, see Methods), statistical associations remained essentially the same, and there were no interactions between age and the tested independent variables.

The abundance of the next most prevalent class of sublimons (the 7.4 kb deletion) was studied in a randomly chosen subset of the individuals. The 7.4 kb deletion (mean 3.47 copies/cell, SD 4.16), where present, was found always as a single species, without the break-point heterogeneity characteristic of the 3.75 kb sublimon class. The abundance of the two sublimon classes was closely correlated in the 26 individuals compared (Fig. 14, Pearson's correlation coefficient $r=0.81$ on the square-root-transformed data as plotted). This points out that the abundance of the most prevalent sublimon class is a good measure of the overall abundance of sublimons in any given specimen. A typical 'rare' sublimon also studied by fluorescent PCR was undetectable in all but a handful of cases, where it was found close to the detection limit of 0.1 copies per cell (data not shown).

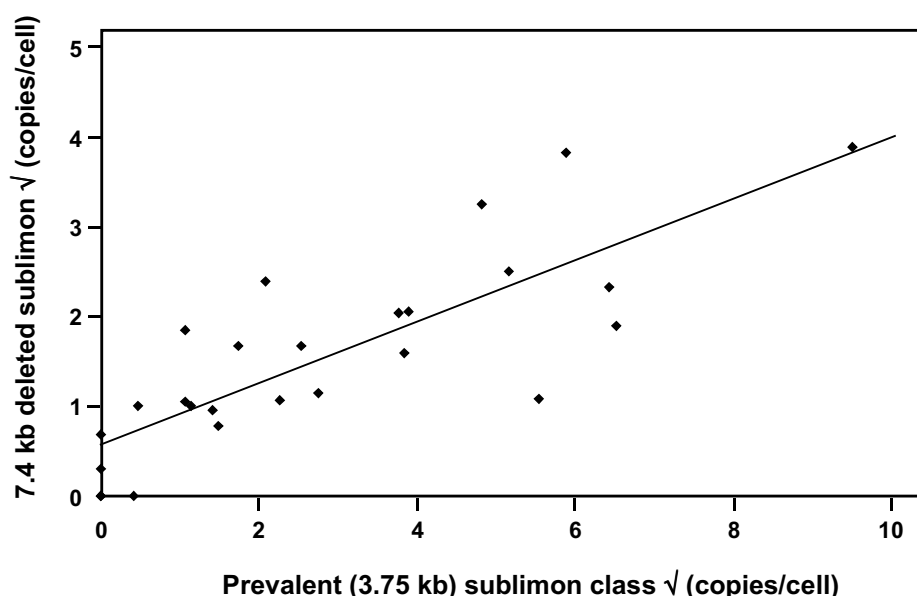


Figure 14. Correlation between the copy numbers of the two most prevalent myocardial sublimon classes. DNA from 26 randomly chosen individuals was used to quantitate the 7.4 kb deletion, which is shown plotted against that of the most prevalent (3.75 kb) sublimon class in the same specimens.

9. Polymorphisms of the candidate genes (unpublished results)

The frequencies of the allelic variants are presented in table 12. No men with the inactive mutant *ALDH2:2* allele were found among the genotyped 198 cases in the series B, all subjects were homozygotes for allele 1. Regarding the studied polymorphisms of *AHD2*, *ALDH2* and *CYP2E1* *Mst1*, *Rsa1* and *Pst1*, either all men or nearly all (excepting 1 to 6 individuals) had the common genotype of *ADH2* and *CYP2E1* *MspI*, *PstI* and *RsaI* polymorphisms in the series, and further statistical analyses were not possible to conduct with these variants. The allele frequencies of *ACE* I/D, *AT1R* 1166A/C and *CYP11B2* -344C/T - polymorphisms are in line with earlier studies of Finnish populations (Kupari et al., 1994, Pastinen et al., 1998, Kupari et al., 1998).

For the association analyses (see Table 13) the men were classified into three groups, with daily alcohol doses of <72, 72-180 or >180 grams/day. The rationale for these values comes from analyses of the heart dimensions, which showed no significant changes before a daily alcohol dose of 72 g, and different effects of heavy (72-180 g/day) and very heavy (>180 g/day) consumption. Consistent with the previous analyses (studies I and II), heart weight and RV cavity size were most constantly associated with the daily alcohol consumption level (Table 13). The subjects homozygous for the rarer alleles were combined with the heterozygotes, except for in the case of *ACE* I/D and *CYP11B2* -344C/T polymorphisms, where all genotypes were analysed as separate groups.

The results of ANCOVA are summarized as F-ratios in Table 13. The independent effects of alcohol were best seen in the right ventricular cavity area and in the total heart weight; both increased with increasing alcohol consumption (data not shown). The polymorphisms of *AT1R* and *AHD3* influenced heart weight and RV cavity area, respectively, in ANCOVA (Table 13). There were no statistically significant interactions between daily alcohol dose and any of the polymorphisms on any cardiac dimension studied.

Table 12. Frequency of the allelic variants of the candidate genes.

	Allele frequency	Genotype frequency	N
<i>ACE</i> I/D	I: 0.61, D: 0.39	II 0.47, ID 0.28, DD 0.25	300
<i>AT1R</i> 1166A/C	A: 0.79, C: 0.21	AA 0.62, AC 0.34, CC 0.04	650
<i>CYP11B2</i> -344C/T	T: 0.49, C: 0.51	TT 0.25, CT 0.48, CC 0.27	679
<i>ADH2</i> <i>MaeIII</i>	1: 0.99, 2: 0.01	1:1 0.99, 1:2 0.01	198
<i>ADH3</i> <i>SspI</i>	2: 0.56, 1: 0.44	2:2 0.30, 2:1 0.51, 1:1 0.18	328
<i>ALDH2</i> <i>EcoRI</i>	1: 1.00	1:1 1.00	198
<i>CYP2E1</i> <i>DraI</i>	d1: 0.88, d2: 0.12	d1d1 0.76, d1d2 0.23, d2d2 0.01	676
<i>CYP2E1</i> <i>MspI</i>	w: 0.98, m: 0.02	ww 0.97, wm 0.03	198
<i>CYP2E1</i> <i>PstI</i>	w: 0.99, m: 0.01	ww 0.98, wm 0.02	199
<i>CYP2E1</i> <i>RsaI</i>	w: 0.99, m: 0.01	ww 0.97, wm 0.03	199

Table 13. The F-ratios from ANCOVA describing both the independent effects and the interaction of alcohol dose and various genotypes on cardiac measurements

		<i>ACE</i> I/D	<i>AT1R</i> 1166A/C	<i>CYP11B2</i> -344C/T	<i>ADH3</i> <i>SspI</i>	<i>CYP2E1</i> <i>DraI</i>
<i>N</i> §		104	352	369	184	352
Heart weight	<i>Genotype</i>	NS	4.31*	NS	NS	NS
	<i>Alcohol dose</i>	NS	3.38*	NS	4.39*	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Left ventricular						
Weight	<i>Genotype</i>	#	NS	NS	NS	NS
	<i>Alcohol dose</i>	#	NS	NS	NS	NS
	<i>Interaction</i>	#	NS	NS	NS	NS
Cavity area	<i>Genotype</i>	NS	NS	NS	NS	NS
	<i>Alcohol dose</i>	NS	NS	NS	NS	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Anterior wall	<i>Genotype</i>	NS	NS	NS	NS	NS
	<i>Alcohol dose</i>	NS	NS	NS	NS	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Posterior wall	<i>Genotype</i>	NS	NS	NS	NS	NS
	<i>Alcohol dose</i>	NS	NS	NS	NS	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Wall/cavity -ratio	<i>Genotype</i>	NS	NS	NS	NS	NS
	<i>Alcohol dose</i>	NS	NS	NS	NS	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Right ventricular						
Weight	<i>Genotype</i>	#	NS	NS	NS	NS
	<i>Alcohol dose</i>	#	NS	NS	NS	NS
	<i>Interaction</i>	#	NS	NS	NS	NS
Cavity area	<i>Genotype</i>	NS	NS	NS	6.24*	NS
	<i>Alcohol dose</i>	4.16*	6.84**	4.75**	4.75*	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS
Anterior wall	<i>Genotype</i>	NS	NS	NS	NS	NS
	<i>Alcohol dose</i>	NS	NS	NS	NS	NS
	<i>Interaction</i>	NS	NS	NS	NS	NS

This analysis shows results after adjustment for age, body size, coronary artery stenosis, cigarette consumption, hypertension and diabetes. According to the calculated daily alcohol dose, subjects were divided into groups of <72 , 72-180 and >180 grams/day. §Number of cases with complete covariate data.

P values from ANCOVA: *P<0.05, **P<0.01.

#Not available, NS, non-significant.

DISCUSSION

1. AHMD among autopsied middle-aged men (I, II)

One aim of this study was to investigate the alcohol-related myocardial abnormalities among autopsied middle-aged men. In the present study, such cardiac abnormalities had actually been detected in a considerable fraction of all the cases at autopsy, usually described by unspecific terms hypertrophy, dilatation, enlargement of the heart or its ventricles, myofibrosis or unknown heart muscle disease. As for most of these cases alcohol was either not present or its effects were mixed with other cardiovascular disease, most commonly coronary heart disease, definite diagnoses of alcoholic cardiomyopathy were rare. Thus further studies were required to substantiate the diagnosis of alcoholic cardiomyopathy. In particular, additional data on the cardiac pathology were needed. This was accomplished in the present study by quantification of both alcohol consumption and cardiac anatomy, combined with data collection on possible confounding factors.

The measurements of the left ventricle (LV) at autopsy showed results comparable to some earlier studies of chronic alcoholics. At increasing alcohol consumption the LV cavity size of the men studied reduced and the wall thickness/cavity size ratio increased. Echocardiographic studies from Finland have shown that chronic alcoholics have increased LV mass and increased LV wall thickness/diameter ratio, compared with teetotalers and occasional drinkers (Kupari et al., 1990, 1991, 1992). Therefore, both this study and the previous echocardiographic studies show LV concentric change at increasing alcohol doses and in addition increased LV mass, which in our series was seen when the whole population of men was analyzed. Notably, most of the earlier studies (Spodick et al., 1972, Ahmed et al., 1980, Askanas et al., 1980, Kino et al., 1981, Mathews et al., 1981, Silberbauer et al., 1988, Urbano-Marquez et al., 1989) have been conducted in relatively small, selected populations of alcoholic patients, where the full exclusion of concomitant coronary artery disease has not been possible. In addition, only a minority of earlier studies has aimed at recording the independent effects of alcohol by rigorous controlling of the confounding factors or multivariate analysis of the factors known to determine the LV dimensions (Kupari et al., 1991).

Although it was not possible for us to estimate the lifetime cardiac function of the study subjects, the detected concentric remodeling of left ventricle in moderate and heavy drinkers is compatible with lifetime diastolic dysfunction due to a stiff left ventricle. A mild to moderate diastolic dysfunction has, indeed, been a commonly encountered abnormality among asymptomatic alcoholics (Lundin et al., 1986, Silberbauer et al., 1988, Kupari et al., 1990, 1991, 1992). Acutely, alcohol decreases the preload of the heart, but apart from that has little or no effect on diastolic function of nonalcoholic subjects (Kupari et al., 1990b). Diastolic dysfunction may thus be the first sign of AHMD (Silberbauer et al., 1988, Kupari et al., 1990, 1991) and tell something about the mechanisms by which alcohol damages the

heart. In animal studies, diastolic dysfunction has also been shown to occur and to precede the development of systolic dysfunction or ventricular dilatation (Thomas et al., 1980). The explanation for this might be an increase in the fibrous collagen content, as observed in the canine heart muscle (Thomas et al., 1980). Increased myocardial fibrosis has been frequently reported also in human subjects with alcoholic heart muscle disease (Ferrans et al., 1965, Hibbs et al., 1965, Alexander, 1966, Bulloch et al., 1972), but being an unspecific response in various disease states of the heart, its relation to the alcohol exposure has remained unclear. Whether there exists a relationship between the amount or distribution of the myocardial fibrosis and alcohol exposure needs to be studied further. The consequence of the structural changes may be a reduction of the uptake of calcium ions from the sarcoplasm into the sarcoplasmic reticulum, as observed earlier (Bing, 1982, Noren et al., 1983), which has the ability to impair the release of myosin head from actin and reduce the rate of the diastolic active relaxation of the ventricles. Furthermore, the altered macroscopic structure itself, possibly due to increased fibrosis, can mechanically impair ventricular filling (Brutsaert et al., 1985).

The relationship between right ventricular size and alcohol use seen in our series is a novel finding. Mostly because of methodological difficulties, earlier studies have not gained information on the right side of the heart (Spodick et al., 1972, Ahmed et al., 1980, Askanas et al., 1980, Kino et al., 1981, Mathews et al., 1981, Lundin et al., 1986, Kelbaek et al., 1987, Silberbauer et al., 1988, Urbano-Marquez et al., 1989, Kupari et al., 1990, 1991, 1992, 1993). In contrast to the echocardiographic studies, the right ventricle is readily accessible at autopsy. We found RV size to correlate independently with the daily alcohol dose in a linear fashion among all subjects. Amongst the non-coronary subpopulation the relation was non-linear. The observed RV dilatation and the increase in LV size from its trough values may both be signs of systolic dysfunction as a result of alcohol toxicity. Increased RV afterload due to passive pulmonary hypertension resulting from LV dysfunction may have contributed to the RV dilatation. There exists also experimental data suggesting that alcohol may directly elevate pulmonary artery pressure (Kettunen et al., 1983, 1992) and furthermore, unexpected pulmonary hypertension has been encountered also in human alcoholics (Koskinen et al., 1986). Recently RV size and function have been noticed as important prognostic factors in idiopathic dilated cardiomyopathy (Gavazzi et al., 1997, Juilliere et al., 1997, La Vecchia et al., 1999). Future clinical studies are needed to explore if RV size or function has comparable significance in alcoholic heart muscle disease.

2. Traditional cardiovascular risk factors and AHMD (I, II)

The left ventricular concentric remodeling found in moderate and heavy drinkers resembles changes observed in ageing of normotensive individuals (Ganau et al., 1995). In contrast, in our series, consisting of middle-aged men, age correlated weakly negatively with LV wall/cavity area -ratio, resembling eccentric remodeling. Age had a positive linear correlation with total heart and RV weight and non-linear regression analyses revealed an

additional positive effect on LV cavity size. LV weight on the other hand was highly dependent on body size, severity of coronary artery disease (CAD) and hypertension, but not on age. These logical relationships support the validity of the cardiac measurements and show that the effect of alcohol is independent of traditional cardiovascular risk factors.

Hypertension has been shown to be commonly associated with concentric LV remodeling (Verdecchia et al., 1995, Heesen et al., 1997), which has also adverse prognostic significance (Verdecchia et al., 1995). We found the history of hypertension to be associated with LV hypertrophy, but not with increased LV cavity size. It has been suggested that some of the effects alcohol on myocardium might be mediated via elevated blood pressure. In our series adjustment for the lifetime history of hypertension did not alter the results. However, the role of hypertension as a mediator or modulator of alcohol's effects on myocardium can probably be complex to be revealed without data on the actual blood pressure values of the alcoholics. In addition, unexpectedly the history of diabetes did not have independent effects on any cardiac measurement tested.

Severity of CAD is a major determinant of left ventricular anatomy. In the present study, both the maximal coronary stenosis and the number of significantly affected vessels also correlated positively with LV size and weight. LV concentric remodeling due to alcohol was found to be highly dependent on the severity of concomitant CAD. The results may be of importance because earlier studies in alcoholic populations have mostly excluded individuals with signs or symptoms of CAD (e.g. Askanas et al., 1980, Kupari et al., 1990) and thus may have missed the interaction. Our results showed, that LV concentric remodeling, occurring dose-dependently in moderate and heavy drinkers, was seen only among men with mild or absent CAD. Men with moderate CAD had essentially similar LV dimensions in all alcohol groups, whereas the combination of severe CAD and heavy or very heavy alcohol consumption associated with slight LV cavity dilatation and increase in LV wall/cavity area - ratio. This supports the idea of LV concentric remodeling being a typical structural manifestation of non-ischemic alcohol-related heart muscle disease (Kupari et al., 1990, 1991). However, already by definition AHMD is a disease affecting individuals without any other significant systemic or heart disease. Of note, already moderate coronary artery disease, easily overlooked in clinical studies without performance of coronary angiography, almost completely abolished the alcohol-related LV remodeling in our series.

What could be the factors involved in the different responses of the coronary and non-coronary subpopulations ? If concomitant CAD is present, acute alcohol exposure has been reported to exaggerate exercise-related ischemia (Orlando et al., 1976, Rossinen et al., 1996) and cause mild impairment or no change in LV function (Gould et al., 1972, Kelbaek et al., 1988). Further supporting its harmful effects, moderate and heavy alcohol use have recently been shown to increase the overall mortality and marginally increase the risk of fatal cardiac events in the presence of clinically established CAD (Shaper and Wannamethee, 2000). The same study showed, however, although based on a small subgroup of cases, that in men without a previous myocardial infarction there was no adverse effects on mortality from moderate/heavy drinking. In men with diagnosed CAD, in contrast to the general population, even light drinking did not offer any protection from death of cardiovascular or other cause

(Shaper and Wannamethee, 2000). Another large prospective study has shown that also among men with previous myocardial infarction, small amounts of alcohol (two to four drinks per week) associate with decreased total mortality (Muntwyler et al., 1998). Thus the relationship between CHD, alcohol and mortality is not clear.

The mechanisms of the interaction between CHD and alcohol have remained unclear. Superimposed effects of ischemia and alcohol toxicity certainly offer greater potential for myocardial damage leading to ventricular dilatation and dysfunction than either of the factors alone.

3. Dose-dependency and reliability of the results (I, II)

Whether a dose-injury relation exists for the long-term effects of alcohol on cardiac structure has remained unclear. Although studies from one center have suggested a linear relationship between total lifetime alcohol consumption and the degree of LV hypertrophy and systolic dysfunction (Urbano-Marquez et al., 1989), other comparable works have failed to reproduce the results. Any clear dose-dependency, linear or other, has been difficult to show in studies done by several groups around the world (Askanas et al., 1980, Kino et al., 1981, Kupari et al., 1991). By re-interpretation of the earlier results of Urbano-Marquez et al. (1989), Kupari et al. (1991) suggest no relation of the LV measurements to ethanol use up to a lifetime cumulative dose of 20 kg/kg (e.g. for a 70 kg individual, this means ca. 16 drinks or one bottle of spirits per day for 20 years) of body weight, and after that only an increase in LV mass and a decrease in ejection fraction. Most of the study subjects thus did not show any dose-dependency and, in addition, some selected subjects forming the results had severe cardiac failure (Urbano-Marquez et al., 1989), making it hard to generalize the results. When effects of duration of the heavy drinking have been studied, significant changes in the LV dimensions or function could be seen only after approximately 12 years of heavy alcohol use (Kupari et al., 1991). In another study, duration of alcoholism (<15 or ≥15 years), not accounting for the quantity of daily consumption, did not correlate with LV mass or systolic function (Askanas et al., 1980).

The results of our study support the view of a complex relationship between the cardiac measurements and alcohol use, and we suggest that alcoholic heart muscle disease can in addition be manifested differently in the left and right ventricle. In fact, the dose-dependency that was found in our series, resembles to some degree the earlier echocardiographic results in Finnish alcoholics (Kupari et al., 1991). Both our data and that on the heavy drinkers by Kupari et al. (1991) clearly show that the average LV size does not increase in correlation to the alcohol use at any level or duration of the intake. The total heart weight in our study and LV mass in the heavy drinking alcoholics (Kupari et al., 1991) correlated in a linear or curvi-linear fashion with increasing alcohol exposure. LV weight among our autopsied subjects correlated non-linearly with the daily alcohol dose, showing first an increase at moderate doses, and then again among the heavy alcoholic drinkers,

consistent with earlier studies in Finnish alcoholics in comparison with controls (Kupari et al., 1990, 1991).

The U-shaped form of the relation of LV cavity and daily alcohol dose is a novel finding. Earlier studies may have missed this because they have usually included only individuals from the two extremes of the spectrum of habitual alcohol consumption (alcoholics and controls). (Spodick et al., 1972, Askanas et al., 1980, Mathews et al., 1981, Kupari et al., 1990, 1991, 1992).

The dosage at which RV dilatation was observed (> 180 g/day) was similar to that at which LV cavity size was seen to 'return' towards normal, and the concentric remodeling was absent. In non-linear analyses among all men there were no effects on RV size before the dose 180 g/day, whereas in the non-coronary subpopulation the relation to alcohol was more U-shaped. As in the case of the left ventricle, earlier studies have not studied all possible levels of daily alcohol consumption, and have not been able even in theory to evaluate the structure of the right ventricle because of its unaccessibility by non-invasive methods.

The questionnaire used to collect data on alcohol consumption for calculation of the average daily alcohol consumption has been validated earlier (Karhunen and Penttilä, 1990). The daily alcohol doses based on the post mortem alcohol reports were found to be in good correlation with both blood and urine alcohol concentration at the time of death, and also with the occurrence of fatty liver and chronic pancreatitis (Karhunen and Penttilä, 1990). The series of autopsies of this study has previously been used to study the incidence of alcoholic liver disease (Savolainen et al., 1993) and disorders of spermatogenesis (Pajarinen et al., 1997). The calculated doses of habitual alcohol consumption, which were used also in the present study, showed good correlations with the incidence of fatty liver and cirrhosis pathology (Savolainen et al., 1993) as well as the spermatogenetic disorders (Pajarinen et al., 1997), revealing for both a threshold at ca. 40 g/day. Furthermore, alcoholic cirrhosis and hepatitis were absent among individuals consuming less than 40 g per day (Savolainen et al., 1993), arguing against significant misclassification of heavy drinkers as teetotalers or light drinkers.

Fixation of the heart either in the systolic or diastolic phase is a factor causing increased variance in the measurements. Earlier it has been shown that the post mortem volumes of the ventricles depend on the stage of rigor mortis and the use of formalin fixation (Sairanen, 1985). Since our series of hearts was studied without fixation and during a relatively short period of time after death, the post mortem changes are considered random. The validity of the cardiac measurements is also supported indirectly by statistically significant and medically plausible associations between body size, age, CAD and hypertension and these measurements. By the time of the autopsy, on average most of the myocardial rigor mortis has already been relaxed, and does even more because of the manipulation of the heart before the measurements. Notably, the *post mortem* delay did not correlate with any of the cardiac measurements.

4. mtDNA rearrangements in normal and diseased myocardium (III, IV, V)

Earlier studies on humans (Hibbs et al., 1965, Tsiplenkova et al., 1986, Sudarikova et al., 1997) and experimental models (Burch et al., 1971, Segel et al., 1975, Mattfeldt et al., 1980, Morvai and Ungvary, 1987, Cunningham et al., 1989) have pointed out the possibility that ingestion of alcohol might damage mitochondria. The present study did not show any association between mtDNA rearrangements and AHMD. By the use of LX-PCR we found numerous 'deleted' PCR products from both controls and cases with alcoholic cardiomyopathy diagnosed at autopsy. The existence of such variant forms of mtDNA in human tissues is widely supported by earlier studies (Remes et al., 1994, Melov et al., 1995, Liu et al., 1998), their significance remaining unproven for any disease or trait studied. Association of acquired myocardial disease with mtDNA rearrangements has been suggested in a few studies (Ozawa et al., 1990, Suomalainen et al., 1992, Bobba et al., 1995, Li et al., 1995, Marin-Garcia et al., 1996). Because the aberrant forms of mtDNA have been found to be present in small amounts in comparison with intact mtDNA in even the worst affected tissues, and since the deletions have been found in apparently equivalent amount in control myocardium (Remes et al., 1994, Lightowers et al., 1999), their significance has remained controversial. In the studies of mtDNA rearrangements by LX-PCR and southern blotting we did not find any systematic differences, neither quantitative nor qualitative, between controls and cases with alcoholic cardiomyopathy diagnosed at autopsy.

At least two different conclusions can be drawn from the results concerning the involvement of mtDNA in alcoholic cardiomyopathy. Firstly, large scale mtDNA rearrangements are highly unlikely to be a primary cause of alcoholic cardiomyopathy, and their role as susceptibility factors of any kind must be a minor one at most. Secondly, the 'deleted' forms of mtDNA were not increased in the myocardium, which questions the hypothesis of increased oxidative stress as a consequence for increased somatic mtDNA deletions as a common pathogenic mechanism for myocardial disease. The amounts of rearranged mtDNA reported in some isolated cases of myocardial disease (e.g. Ozawa et al., 1990) have been comparable to those seen in normal aging. While initial reports showed decreased activities of respiratory complexes by age (Trounce et al., 1989, Yen et al., 1989), more recent studies have failed to show any clear age-dependent respiratory decline (Brierly et al., 1997, Chretien et al., 1998). Alcoholics with subtle cardiac dysfunction but without clinical symptoms or cardiomyopathy were recently shown to have normally functioning respiratory complexes (Miro et al., 2000), as were muscle mitochondria from myopathic chronic alcoholics (Cardellach et al., 1992). It has been suggested that studies of particular mtDNA deletions would underestimate the abundance of deleted molecules in the tissues and that the collective amount of the different variant forms would be significant (the so called 'tip of the iceberg' –hypothesis) (Cortopassi et al., 1992). The results, that we have obtained here, in the particular case of alcoholic cardiomyopathy, do not support this as most of the theoretical rearrangements were covered by the LX-PCR approach used, and still no increase in the aberrant forms was seen. Another theory is that an uneven tissue distribution of the abnormal mtDNAs could lead to impairment of oxidative phosphorylation in certain cells or

parts of the tissue, for which there is some evidence (Muller-Hocker et al., 1993). This would be harder to exclude in the case of alcoholic cardiomyopathy and would need multiple sampling and rigorously controlled quantification of the deletions in different parts of the heart.

In the present studies, the LX-PCR methodology commonly used to study mtDNA rearrangements in relation to disease proved to be highly sensitive. Although pathological mtDNA deletions have been reliably detected by LX-PCR (Kleinle et al., 1997), the presence of aberrant mtDNA forms accounting for only a minor part of the pool of mtDNA molecules seems to be more difficult to study. We found that rearranged mtDNA molecules present at only one or a few copies per cell are easily detected by standard LX-PCR methodology. Like any other PCR -based method, LX-PCR detects shorter molecules preferentially, overestimating their abundance in relation to the longer template molecules and thus giving a qualitative rather than quantitative profile of the mtDNA molecules in a sample. In addition, we found it possible to obtain variable amounts of deletion-derived LX-PCR products in various samples simply by varying the template concentration in the PCR reaction. Combined with the sensitivity of the method this creates a risk of detecting 'differences' in the representation of deleted mtDNA between samples where none, in reality, exist. The significance of earlier reports linking mtDNA deletions to aging and acquired myocardial diseases is questionable and needs to be critically re-evaluated in the light of the present results.

5. mtDNA sublimons offer a novel pathogenic mechanism for mitochondrial disease (III, IV, V)

During the present series of studies the existence of variant mtDNA forms in different tissues began to attract more attention, mostly because of its potential significance and importance as a more universal phenomenon in mitochondrial genetics. To describe these consistently encountered molecules, the term sublimon was adopted from plant mitochondrial genetics. This was because of the apparent similarities between the rearranged human mtDNA molecules and those present at higher levels in cases of frank mitochondrial diseases. In plants, sublimons are rearranged mtDNA molecules found in healthy individuals at very low levels, but at high amounts in strains exhibiting a mitochondrial cytopathic condition called cytoplasmic male-sterility (CMS) (Small et al., 1989). The genetic defects causing some of the human mitochondrial diseases associated with multiple deletions of mtDNA (MNGIE and one form of adPEO) have recently been clarified (Nishino et al., 1999, Kaukonen et al., 2000), showing the potential of impaired mtDNA replication or maintenance as a cause of disease.

The structures of sublimons found in seemingly healthy tissues of the individuals studied have striking similarities with the pathological deletions. Many of the sequence break-points found in our control subjects correspond precisely or almost precisely with pathological deletions reported earlier (Moslemi et al., 1996, Kleinle et al., 1997, Moslemi et

al., 1999). A hotspot region for deletion breakpoints in multiple deletion disorders, such as adPEO, MNGIE and IBM (Zeviani et al., 1989, Moslemi et al., 1997, 1999, Jansson et al., 2000), was involved in a majority of the sublimon breakpoints of our control heart. This so called common breakpoint region (CBR) is located near to the end of the D-loop around nt 16070 of the human mtDNA sequence, preserving O_H in almost all of the sublimons detected. Unlike the deletions in the pathological states, the sublimons in control heart mostly had O_L removed by the rearrangement, creating a molecule unable to replicate by the commonly adopted mechanism. Although this can partly be assigned to the methodology used, which preferentially detects shorter sublimons, it also suggests that there might exist alternative ways of mtDNA replication. Like the mtDNA deletions in pathological states (Poulton et al., 1993, Moslemi et al., 1996), sublimons in control heart can exist in partially duplicated and triplicated forms, and also as deletion mono- and multimers.

There are several theoretically possible mechanisms, which might generate and/or maintain low levels of sublimons in normal tissues. The H-strand replication beginning at O_H, proceeding around the genome and pausing at the end of the D-loop, might be followed by continued DNA synthesis after strand invasion elsewhere in the genome. These events would finally create either a deleted or partially duplicated mtDNA molecule. Thus the various forms of sublimons detected can either be the initial products of aberrant mtDNA replication (Shoffner et al., 1989, Baumer et al., 1994) or on the other hand can be formed from each other by homologous recombination (Holt et al., 1989, Holt et al., 1997) between the rearranged molecules and the intact genome. AT-rich sequences have also been noted in the vicinity of human mtDNA deletion break-points (including the 'common deletion'), which potentially promotes strand separation and also the formation of bent DNA in these regions (Hagerman, 1990). Interestingly, the second most commonly observed breakpoint region in our study lies within the gene for tRNA-leu(UUR), which is adjacent to the binding site for mTERF (Kruse et al., 1989, Fernandez-Silva et al., 1997). The termination associated sequence (TAS) -region of the D-loop is also one of the protein-binding sites of mtDNA. Interaction between mtDNA binding proteins is an attractive mechanism that may be involved in the generation of sublimons.

The documentation of sublimons in different human tissues of non-diseased individuals requires us to re-evaluate thinking about the pathogenic mechanisms by which mtDNA rearrangements lead to disease. Formerly it has been thought that the rearrangement(s) must be created by a single mutational event, followed by clonal expansion (Brown and Wallace, 1994) because of replication advantage or some selective force acting on the aberrant form(s). Instead of the involvement of individual mutational events, the material for clonal amplification seems to exist in (almost) all healthy tissues, and multiple deletions found in mitochondrial disorders most probably represent illegitimately amplified sublimons. This approach gives us at least three separate mechanisms for the generation of significant amounts of mtDNA rearrangements found in pathological states. 1) Enhancement of the process(es) which continuously generate sublimons, 2) inhibition of the process(es), by which sublimons are usually lost and 3) a (possibly transient) alteration in the selective value of sublimons, either at the level of phenotype or replicative advantage.

6. The relationship of sublimons, disease and aging (unpublished data, see appendix)

It was shown in studies III-V that myocardial sublimons resemble closely aberrant molecules of pathological states. Since there have been claims about the causal involvement of somatic mtDNA deletions in aging (Cortopassi et al., 1992, Nagley and Wei, 1998), and also in acquired myocardial disease (Ozawa et al., 1990, Bobba et al., 1995, Marin-Garcia et al., 1996), it was interesting to study whether sublimons accumulate under certain states in the heart. The prevalent class of sublimons, studied by semi-quantitative PCR in the series of 300 autopsied males, was found to vary in abundance by three orders of magnitude in heart. The amount of sublimons (as copies/cell) was not associated with any myocardial pathological feature or cardiac dimension, but correlated independently with age and intensity of life-time smoking. Although age was strong and independent factor determining sublimon abundance, accumulation of sublimons was not a necessary concomitant of aging, since high sublimon amounts were encountered among individuals of all ages. An environmental factor, tobacco smoking was also a weak, but statistically significant determinant of sublimon levels, suggesting that environmental factors may play some role in accumulation of sublimons under certain circumstances. Smoking has been suggested to associate with increased amounts of mtDNA damage and increased prevalence of the 'common 4977 bp' or other mtDNA deletions (Ballinger et al., 1996, Fahn et al., 1998, Lee et al., 1998). Pack-years of cigarettes smoked has been suggested to be independently associated with a 4839 bp mtDNA deletion, but not with the 4977 bp deletion, which was determined solely by age (Fahn et al., 1998). Unfortunately the methods used to quantitate 'mtDNA damage' (Ballinger et al., 1996) have recently been shown to be poorly quantitative and prone to various artefacts (Lightowers et al., 1999). Concerning the relationship between sublimons and AHMD in the present study, the intensity of alcohol drinking did not correlate with the prevalence of the common class of sublimons studied.

The abundance of sublimons in heart did not associate with any common myocardial pathological feature or the cardiac dimensions. According to our results, it is evident that the interindividual variation in sublimon levels lacks phenotypic correlation, but could the low levels of aberrant mtDNA forms be expected to account to any significant phenotype in any case? It is known that in cell culture models of the diseases, re-arranged mtDNA forms must represent at least 60% of total mtDNA in order to impair OXPHOS function (Hayashi et al., 1991a). Although there is no method to reliably estimate the 'total sublimon load' of a tissue, we see it as highly unlikely, based on previous experiments by us and others that all sublimons together could represent more than ca. 10% of wild-type mtDNA in any tissue studied. In most cases, sublimons most probably represent much smaller fractions of the total mtDNA pool. A post mortem study reported approximately 15% and 35 % of deleted mtDNAs in two adPEO patients, yet without consistent myocardial pathological findings (Suomalainen et al., 1997). The amounts of wild-type and deleted mtDNA have not been

reported for arPEO, a mitochondrial disease with multiple mtDNA deletions, which is associated with severe cardiomyopathy (Bohlega et al., 1996).

The observed heterogeneity of the rearrangement break-points, and the microscale heterogeneity in some hotspot regions suggests the existence of at least 50-100 and maybe several hundreds of different sublimon species in heart. Large number of different aberrant mtDNA species has earlier been used as an argument for the functional significance of age-related mtDNA deletions (the ‘tip of the iceberg’ –hypothesis) (e.g. Soong et al., 1992, Cortopassi et al., 1992). Although respiratory chain function has initially been claimed to decline dramatically during aging (Trounce et al., 1989, Yen et al., 1989), more recent studies have shown no correlation between age and respiratory decline (Chretien et al., 1998, Brierly et al., 1997). Most probably the age-related changes observed in some studies are due to altered physical activity or some underlying non-mitochondrial disease, as suggested earlier (Brierley et al., 1996), and the development of heart pathology requires considerably higher relative amounts of mutant mtDNA.

We performed an estimation of sublimon abundance in aging human heart by extrapolating sublimon copy numbers and taking into account a considerable amount of interindividual variation (Figure 15). It seems that sublimon copy number per cell would not reach any threshold level capable of causing pathological consequences during a realistic human life-span. Even if we would consider a more exponential rate of sublimon accumulation after the age of 70 years, phenotypically potentially meaningful sublimon levels would be awaited to be an extreme rarity, even among centenarians. The factors influencing the rate of sublimon accumulation among the oldest old are, however, not known and a detailed study over the whole human life-span could yield additional information. The rate of sublimon accumulation would anyway need to be exceptionally high at some point of the late life in order to reach the threshold before age of 200 years.

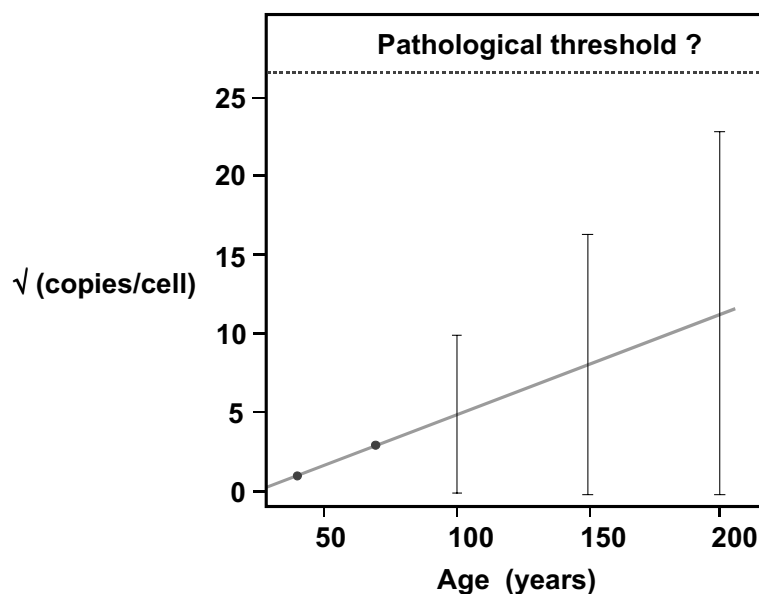


Figure 15. Extrapolation of sublimon levels in aging human heart represented by the average levels and the considered variation.

Finally, abundances of the two most prevalent sublimons in the heart, the 7.4 kb deletion and the 3.75 kb sublimon class, correlated well with each other. Thus the abundance of the most common 3.75 kb sublimon class seems to be a good measure of the sublimon load in a given tissue. Other sublimons studied using a similar approach were found to be much rarer, being present at most at only 0.1 copies per cell. Since this is near the detection limit of the fluorescent PCR method, further analyses of these sublimon molecules were not done.

7. Genetic association studies (unpublished results)

The role of chosen genetic variants in the development of AHMD was screened by searching for alcohol-by-genotype interactions on the various cardiac dimensions. Each of the tested genes had a biological relevance to AHMD, but their importance as susceptibility factors for AHMD had not been assessed before. Since frequencies of the mutant alleles of the functional polymorphisms of *ADH2* and *ALDH2*, as well as the oriental alleles of *CYP2E1*, were extremely low, they were excluded as genetic susceptibility factors for AHMD.

There was an association between *ADH3* genotype and RV size, the carriers of faster *ADH3:1* alleles showing decreased RV size. Based on biochemical studies, *ADH3:2* allele carriers would be expected to have higher tissue ethanol concentrations, and this association could support a direct toxic influence of ethanol on the right ventricle. Instead of an interaction with alcohol, *ADH3* polymorphism had an independent effect, which is hard to explain, however. A modifying effect of the *ADH3* genotype, instead of a direct one, would certainly be biologically more logical. There is always the possibility that this genetic association represents a chance finding due to the relative large number of statistical tests in our work.

Our study failed to identify any interactions with daily alcohol use for the various polymorphisms in the genes encoding enzymes of the RAA -system. Except for the association of cardiac mass with *AT1R* polymorphism, the data on the direct genotype effects were also negative. These results are well in line with earlier clinical studies from Finnish populations showing no associations between either the I/D polymorphism of *ACE* or the 1166A/C polymorphism of *AT1R* and the LV dimensions and mass at echocardiography (Kupari et al, 1994, Reissell et al, 1999). There is, however, controversy between the present data and a previous Finnish observation showing aldosterone synthase -344C/T polymorphism as a significant determinant of LV size and mass (Kupari et al, 1998). The populations in these two studies were remarkably different, however, with the earlier work involving relatively young men and women free of coronary artery disease (Kupari et al, 1998). Recently, a large study reported negative results on the role of aldosterone synthase -344C/T polymorphism in LV structure and function variation (Schunkert et al., 1999).

8. Conclusions and perspectives

The independent effects of alcohol on the heart were shown to be complex and partly non-linear, questioning many earlier assumptions and common beliefs about AHMD. The U-shape of the relationship between LV size and the intensity of alcohol consumption, plus the involvement of the RV, were both novel findings. In addition to alcohol, concomitant CAD was of major importance to the development of AHMD so that alcohol-related ventricular remodeling was limited to the non-coronary subpopulation.

Most human tissues were found to contain a heterogeneous set of rearranged mtDNA molecules, present in small amounts relative to the wild-type mtDNA. By analogy with plant mitochondria, these rearranged mtDNAs were termed sublimons. Sublimons were most abundant in post-mitotic tissues like heart and skeletal muscle and the most abundant sublimon species were found to be distributed in a tissue-specific fashion. Sublimon molecules of control human heart resembled mtDNA rearrangements encountered in pathological states with multiple deletion diseases (adPEO and MNGIE), suggesting sublimon amplification as a potential pathogenic mechanism to yield significant amounts of rearranged mtDNA.

Cellular copy number of the most prominent class of mtDNA sublimons in heart correlated with age and intensity of smoking, but not with any myocardial pathology. It is suggested that considerably larger relative amounts of rearranged mtDNAs apart from those encountered in normal aging, are needed to cause significant tissue dysfunction. This can account for at most only a small fraction of cases of myocardial or coronary artery disease. Thus, we propose that sublimons, instead of being a causal factor in cardiac aging, co-exist with wild-type mtDNA in an equilibrium which is regulated during aging by as yet unknown mechanisms. Since most of the sublimon species are present at fewer than one copy per cell, they can not be inherited, but instead must be generated somatically. The processes of sublimon generation, maintenance and destruction are unraveled and subject for future studies on the pathogenesis of mitochondrial as well as other disease.

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APPENDIX

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The relationship between somatic mtDNA rearrangements, human heart disease and aging

Brief title: mtDNA rearrangements, heart disease and aging

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STRUCTURED ABSTRACT

OBJECTIVES: Our aim was to determine the relationship between rearranged mitochondrial DNA molecules, human heart disease and aging. **BACKGROUND:** The accumulation of low-abundance, somatic mtDNA re-arrangements (sublimons) has been proposed as a potential contributor to cardiac aging and also certain types of myocardial disease. Tissue-specific sublimons, varying in abundance by three orders of magnitude between control subjects, have recently been observed in myocardium, but their relationship with cardiac pathology has remained controversial. **METHODS:** To study specific sublimons in myocardium, a semi-quantitative fluorescent PCR assay was applied on genomic DNA extracted from left ventricle in a series of 300 well-characterized male victims of sudden death. Besides the routine autopsy protocol, cardiac dimensions were measured by planimetry and coronary narrowings via a silicone cast of the arteries. A structured interview of the spouse was conducted to obtain data on previous illnesses and cardiovascular risk factors. **RESULTS:** The most prevalent classes of sublimons were present at <0.1 to 90 copies per cell, but did not correlate with myocardial pathology or cardiac dimensions. In multiple regression analyses age ($\beta=0.43$, $P<0.0001$) and smoking ($\beta=0.25$, $P=0.012$) were independent factors determining sublimon levels. **CONCLUSIONS:** Sublimons accumulate with age in myocardium of a subset of individuals, but to levels where they do not appear to have any phenotypic effects. Sublimon accumulation is also not associated with any common cardiac pathology. We propose that, instead of being a causal factor in cardiac aging, sublimons co-exist with wild-type mtDNA in an equilibrium which is regulated by as yet unknown mechanisms.

Key words: mitochondrial DNA, heart ventricle, aging, cardiomyopathy, smoking

CONDENSED ABSTRACT

We have studied the relationship between low abundance, rearranged mitochondrial DNA molecules (sublimons), cardiac pathology and aging, using a semi-quantitative fluorescent PCR assay applied on myocardial DNA extracted from a series of 300 well-characterized male victims of sudden death. Besides the routine autopsy protocol, cardiac dimensions and coronary narrowings were measured, and a structured interview of the spouse conducted to obtain data on cardiovascular risk factors. The levels of the most prevalent sublimon classes did not correlate with myocardial pathology or cardiac dimensions. In multiple regression analyses age and smoking were independent factors determining sublimon levels.

ABBREVIATIONS

mtDNA	mitochondrial DNA
PEO	progressive external ophthalmoplegia
adPEO	autosomal dominant progressive external ophthalmoplegia
arPEO	autosomal recessive progressive external ophthalmoplegia
KSS	Kearns-Sayre syndrome
PCR	polymerase chain reaction
ANCOVA	analysis of covariance
CAD	coronary artery disease
BSA	body surface area
MNGIE	mitochondrial neurogastrointestinal encephalomyopathy

INTRODUCTION

Human mtDNA is a circular genome of 16568 bp, coding for essential components of the oxidative phosphorylation machinery. Re-arrangements of mtDNA, in the form of deletions and partial duplications, are found in a variety of pathological states often affecting heart and skeletal muscle, as well as other organ systems (1). These pathological mtDNA re-arrangements always co-exist with wild-type mtDNA in variable proportions, a situation denoted as heteroplasmy. PEO (progressive external ophthalmoplegia) with autosomal recessive inheritance and multiple mtDNA deletions is manifested as severe cardiomyopathy of infancy (2). In KSS and Pearson's syndrome, characterized by single, clonal mtDNA rearrangements, heart conduction abnormalities are more often seen, such as various branch blocks, atrioventricular or complete heart block (3, 4). In these disorders the cardiac pathology is typically associated with a considerable mutational load, 10-50 % or more of the total mtDNA being represented by the rearranged molecules.

Based on results obtained by PCR, it has been suggested that deleted mtDNAs accumulate during the course of aging in significant amounts, thus contributing to cellular senescence (5, 6). In addition, some forms of idiopathic cardiomyopathies have been proposed to be associated with mtDNA deletions present in myocardium at low levels, comparable with those detected during normal aging (7-12). An association between deleted mtDNAs and coronary artery disease has also been proposed (13). These reports are all based on studies of relatively few individuals. In recent reports, we and others have used long PCR to detect rearranged mtDNAs even in healthy individuals (14-16). A meaningful disease association of these low abundance rearranged mtDNAs has been questioned by the lack of functional evidence and by the demonstration that long PCR can easily detect deletions present at only one or a few copies per cell (15), i.e. <0.1 % of mtDNA.

Recently, we have characterized the rearranged mtDNAs in human cells and tissues, and detected a specific set of these molecules in control adult heart (16). Because of their low abundance and structural similarity with rearranged molecules found in pathological states, such as adult PEO (17) or the MNGIE syndrome (20), these molecules were termed sublimons, by analogy with such molecules previously reported in plant mitochondria (18). Re-arrangement break points were scattered around the genome, but several hotspot regions were detected, and marked microscale heterogeneity was also observed in specific areas. One particularly prevalent class of myocardial sublimons had heterogeneous break points, respectively, in the region near the terminus of the D-loop (np 16067-16085) and in the gene for tRNA-leu(UUR) (np 3255-3272), generating a 3.75 kb partial duplication interconvertible by homologous recombination with a 12.8 kb deletion. Using semi-quantitative fluorescent PCR, the abundance of this prevalent class of sublimons in heart was found to vary over three orders of magnitude between healthy individuals (16). Another prevalent myocardial sublimon, previously reported as a 7.4 kb deletion (7-12), has break-points at the D-loop terminus as above, and at a 12 bp repeat of the same sequence, located within the gene for ATP synthase subunit 6.

To assess the relationship between these molecules and cardiac pathology, we have now studied a series of 300 autopsied hearts of well-characterized, consecutive male victims of sudden death.

METHODS

The autopsy series

A series of 300 consecutive victims of sudden death, all males (aged 33-69 years, mean 51.2 years, SD 9.6 years), was collected at the Department of Forensic Medicine, University of Helsinki in 1991-92. The cause of death was cardiovascular disease in 39%, other disease in 21% and accidental in 40%. The Ethics Committee of the Department of Forensic Medicine, University of Helsinki, approved the study. Local coronary narrowings were measured from a silicone cast model of the coronary tree (see Ref. 20 for details). The heart and separated ventricles were weighed and cardiac dimensions at the equatorial region of the ventricles drawn into transparent sheets for later analysis by computer-assisted planimetry (for details see Ref. 21). Data on previous illnesses and cardiovascular risk factors was obtained by a personal interview of the spouse or close acquaintance in 147 cases.

DNA extraction and fluorescent PCR

Heart muscle samples from left ventricular myocardium were frozen at $-70\text{ }^{\circ}\text{C}$ and DNA was extracted by standard methods (15). The break-point regions of the two most prevalent classes of sublimons were amplified, as previously (16) by PCR using one fluorescently labelled oligonucleotide primer (np 16153-16133 of the human mtDNA sequence) and one unlabelled primer (np 3150-3168 for the 3.75 kb partial duplication and np 8531-8550 for the 7.4 kb deletion, see Figure 1). For semi-quantitative analysis, multiplex reactions also included primers to amplify a single-copy gene as an internal standard (16). Fluorescent products were analysed by capillary electrophoresis using GeneScan software on an Applied Biosystems 310 Genetic Analyzer, which resolves at the nucleotide level. Sublimon abundances were computed relative to the single-copy standard, under conditions where saturation effects were excluded (for details see Ref. 16).

Statistical analysis

The factors included in the multiple regression analyses (RA) were age, body surface area, smoking (number of cigarettes/day), alcohol consumption (g/day), maximal coronary stenosis, the presence of hypertension and diabetes (both dummy-coded variables) and *post mortem* time. The multiple RA results are given as standardized regression coefficients (β) and *P* values. For ANCOVA, sublimon abundance (copies/cell) was divided into three equally sized groups (N=98 in each) by the 33.33rd and 66.67th percentiles, corresponding to values 1.19 and 4.86 copies/cell, respectively. Age was similarly divided into three equally sized groups of <46, 46-58 and >58 years (N=103, 100 and 97, respectively). Severity of CAD was alternatively classified as the number of arteries having >50% luminal stenosis. Variables with grossly asymmetric distribution were square root- or log-transformed before statistical analyses. *P* values <0.05 were considered as statistically significant. The analyses were made using Statistica/Win (Version 5.0, StatSoft, USA) and SPSS/Win (Version 9.0, USA).

RESULTS

The most prevalent class of sublimons (3.75 kb partial duplication) was found to be present in myocardium in the range 0-90.6 copies per cell (mean 6.1, SD 9.9). In 28 out of the 294 samples successfully analysed, these sublimons were below the detection limit. Mean ages for the three groups (N=98 in each), defined by the 33.33rd and 66.67th percentiles of sublimon abundance, were 48.5, 51.6 and 56.6 years.

In multiple regression analysis (Table 1), when all selected factors were entered into the model (N=101), the number of sublimon copies per cell was independently determined in a statistically significant manner by age ($\beta=0.43$, $P<0.0001$) and also by the number of cigarettes smoked ($\beta=0.25$, $P=0.012$). Forward and backward stepwise analysis methods gave the same significant factors. As is evident from the scatterplot with age (Fig. 2), there was no clear threshold value of age for sublimon accumulation. Furthermore, high copy numbers were seen among subjects of all ages, but some of the oldest subjects also had sublimon levels at or near the detection limit.

In ANCOVA, adjusting for age, the severity of coronary artery disease (CAD) was not associated with sublimon levels ($P=0.89$, N=294). The presence of diffuse interstitial microscopic myocardial fibrosis ($P=0.53$, N=294) or an autopsy diagnosis of dilatation, hypertrophy or other myocardial pathologic finding (cardiomyopathy, fatty infiltration of myocardium) ($P=0.62$, N=294), also had no effect on sublimon copy number. Whether the subject was a smoker or a non-smoker ($P=0.24$, N=139), or had a history of diabetes ($P=0.54$, N=141), was not related to sublimon abundance. In ANCOVA, none of the cardiac dimensions, adjusted for body surface area (BSA) and age, was related to sublimon copy number (divided into three groups, see Methods, data not shown). When all of the analyses were repeated with age as an independent factor (divided into three groups, see Methods), statistical associations remained essentially the same.

Using a similar approach, we compared the abundance of the next most prevalent class of sublimons (the 7.4 kb deletion) with that of the most prevalent (3.75 kb) class, in a subset of the individuals. The 7.4 kb deletion, where present, was found always as a single species, without the break-point heterogeneity characteristic of the 3.75 kb sublimon class (see Fig. 3). The abundance of the two sublimon classes was closely correlated in the 26 individuals compared (Fig. 4, Pearson's correlation coefficient $r = 0.81$ on the square-root-transformed data as plotted, mean ratio of abundances 3.47, SD 4.16). This is consistent with the proposition that the abundance of the most prevalent sublimon class is a good measure of the overall abundance of sublimons in any given specimen. A typical 'rare' sublimon also studied by fluorescent PCR was undetectable in all but a handful of cases, where it was found close to the detection limit of 0.1 copies per cell (data not shown).

DISCUSSION

This analysis covers by far the largest, unbiased set of individuals yet investigated for the relationship between the abundance of rearranged mtDNAs in heart muscle and heart pathology. It demonstrates that inter-individual variation in mtDNA sublimon copy-number in heart does not associate with any common cardiac disease or phenotypic trait studied. This conclusion is clearly at variance with the common view of the published literature concerning both cardiomyopathy and ischemic heart disease. We believe that one explanation for this discrepancy lies in the small number of samples, and especially controls, studied by previous authors. For example, in the report by Kim *et al.* (11), the prevalence of the 7.4 kb deletion was studied in 24 patients with severe dilated cardiomyopathy, compared with just 9 controls. The median age of the controls was also 33 years, whereas that of the patients was 43. Only three controls were above age 40, and all three had detectable levels of the 7.4 kb deletion. From the scatter-plots shown in Figures 2 and 4, it is clear that a minority of individuals, especially in the older age groups, have sublimon levels far outside of the range seen in the majority.

Inclusion of just one or two such individuals in the patient group would be sufficient to shift the mean of the distribution by a considerable amount, whilst having little obvious effect on the standard errors.

Dilated cardiomyopathy requiring heart transplantation is an extreme cardiac condition. When there is such an energetic crisis in the tissue, some compensatory mechanisms would be expected, which could plausibly include mtDNA amplification. This amplification would automatically favour the accumulation of rearranged molecules having replication advantage. However, this does not mean that mtDNA rearrangement is a causal factor in the disease. The tissue composition is also quite different in a highly fibrous, thinned ventricle than in control myocardium. We have shown previously that the quantitative and qualitative representation of sublimons varies between tissues in a systematic manner (16). In any case, this extreme form of cardiac disease affects only a very small minority of individuals, hence there is almost certainly no overlap with our population-based material. Cases of idiopathic dilated cardiomyopathy associated with significant levels of deleted mtDNAs, such as the inherited form described by Suomalainen *et al.* (22), may be very rare.

Similar considerations apply to the report of Corral-Debrinski *et al.* (13), who based their conclusion of an association between mtDNA rearrangements and ischemic heart disease on analysis of DNAs from just five patients. The 7.4 kb deletion was analysed quantitatively in just two patients and no controls, although it was detected qualitatively in each of four controls who were within the same age range as the patients. Pre-selection of a small group of patients, in this case with chronic, diagnosed CAD, may again have biased the findings. The so-called common (5 kb) deletion was reported at elevated levels in these patients, yet in control hearts or even those with severe dilated cardiomyopathy it is present at much lower levels than the 7.4 kb deletion (11), suggesting that tissue changes consequent upon chronic disease or its management could account for the findings in this limited group of patients. In contrast, our unbiased, population-based survey included 100 in whom CAD was the diagnosed cause of sudden death. This allowed for a much more rigorous statistical analysis, which did not support any association between CAD and mtDNA rearrangements. At best, such deletions may be associated with a small subgroup of patients with unusual or extreme forms of heart disease. Even here, causality is doubtful.

Another serious drawback of previously published analyses is the type of quantitation employed (serial-dilution PCR, using primers for a rarely deleted region of mtDNA as a control for 'total' mtDNA). Because mtDNA copy number varies considerably between cell-types, and is also influenced by tissue remodelling in response to external stimuli (23, 24), the use of this standard is also potentially subject to the effects of tissue changes in individuals with chronic disease. Another problem frequently encountered with mtDNA amplimers is cross-reaction with mitochondrial pseudogenes in nuclear DNA (25). Tissue type or quality plus details of sample preparation seem to be major variables influencing the degree to which a given primer pair amplify *bona fide* mtDNA as opposed to nuclear pseudogenes (26-28). Our use of a primer pair for a single-copy nuclear gene as an internal standard for quantitating mtDNA rearrangements avoids these potential artefacts. Our sublimon-specific primers also do not amplify mtDNA-related sequences in nuclear DNA (16).

In multi-variate analyses we found myocardial sublimon abundance to correlate with age and the intensity of lifetime smoking, independently of other cardiac risk factors. Strikingly, whilst some of the older men had elevated levels of sublimons in the myocardium, most had the basal levels of a few copies per cell or less, which also dominated amongst the younger subjects. Sublimon accumulation is

therefore not a necessary concomitant of aging. However, the data for two different rearrangements that we studied are highly concordant, implying that sublimon accumulation, where it occurs, is general, rather than applicable only to specific rearranged species.

The finding of an overall age association is in line with previous studies carried out on many fewer individuals. These earlier studies, using semi-quantitative PCR to detect specific, deleted mtDNA species in heart (5, 13, 29, 30), also found considerable variation in deletion levels amongst subjects of any given age. The amount of variation indicates that factors other than age must be involved in determining cardiac sublimon levels. These may be environmental and/or genetic. Cigarette smoking appears to be one such factor, but alcohol consumption is not. Nevertheless, the lack of phenotypic correlations in our study strongly suggests that the age-associated accumulation of somatic mtDNA rearrangements seen in some individuals is of no pathological significance in regard to cardiac aging, at least where sublimon levels remain within the limits revealed in this study (<100 copies/cell of the prevalent class).

Could sublimons, at the maximum levels observed here in aged subjects, be expected to account for any significant cardiac phenotype? Although there is at present no reliable method to quantitate total sublimon load in any DNA sample, the prevalent sublimon class studied here probably represents a significant fraction of all sublimons present, based on previous analyses (15, 16). Therefore, the total sublimon load is unlikely to exceed 10% of all heart muscle mtDNA in any case studied in the present series. In cultured cell models, deleted molecules must comprise at least 60% of the total mtDNA to produce a marked impairment of respiration (31). In human mtDNA disorders, a mutant gene dosage of at least 10-50 %, usually more, has been observed in clinically affected, post-mitotic tissues. In one *post mortem* study (32), deleted mtDNAs were found to represent approximately 15% and 35% of total heart mtDNA in two adPEO patients, yet were not associated systematically with any specific heart pathology. Cardiac symptoms are generally absent from adPEO patients (M. Zeviani, personal communication), although cardiomyopathy is present in some cases of arPEO (2), where amounts of deleted mtDNA may be even higher. Reports of an age-associated decline in respiratory chain function (33-36) have been used to support the functional significance of small amounts of specific mtDNA deletions. However, more recent results have shown no correlation between age and respiratory decline (37, 38).

In earlier studies, the wide data scatter and low number of subjects studied made it impossible to judge the degree to which sublimon accumulation was a linear function of age, or even to estimate the extent of this variation in a statistically meaningful manner. Our study, based on 300 individuals, now makes it possible to provide at least tentative answers to both questions. After square-root transformation (Fig. 2c), we found sublimon levels within any given 5-year age-class to be distributed about a mean value with an approximately constant standard deviation of slightly less than 100% of the mean. Furthermore, we found that the mean square-root of sublimon abundance increases with age in a plausibly linear fashion. Extrapolating from this analysis, the prevalent sublimon class would reach about 100 copies per cell (some 2% of all mtDNA) in a typical individual at around age 210 years. Even within the tiny subpopulation of individuals who accumulate sublimons at the upper end of the rate distribution, the prevalent sublimons are unlikely to reach a pathologically significant level within any foreseeable human lifespan.

In summary, mtDNA sublimon abundance was unrelated to cardiac pathology among middle-aged

men who had died suddenly. Controlling for potential confounding factors, there was an increase in the level of sublimons with age and with increased smoking. However, there was no support for any causal link between mtDNA rearrangements and myocardial aging. Sublimon involvement in specific, but much rarer forms of myocardial disease states cannot, however, be excluded, based on the present data, and needs further study in the future.

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TABLES AND FIGURES

Table 1

Multiple regression analysis results of potential factors affecting sublimon abundance, given as standardized regression coefficients (β) and P values. N=101.

Model	β	P value
Age	0.43	<0.0001
Smoking intensity (cigarettes/day)	0.25	0.012
Alcohol consumption (grams/day)	-0.14	0.14
BSA	0.12	0.21
<i>Post mortem</i> time	0.09	0.33
CAD*	-0.02	0.85
Diabetes†	-0.001	0.95

*maximal luminal stenosis of any coronary artery

†as recorded by the interview

BSA = body surface area, CAD = coronary artery disease

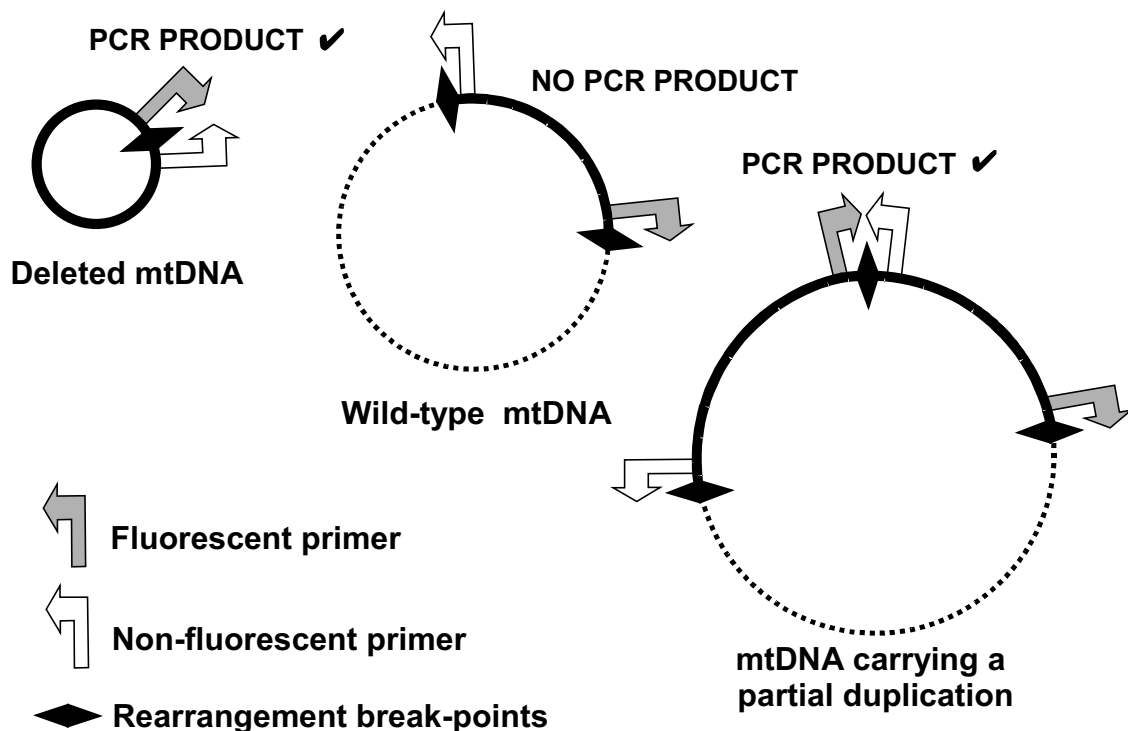


Figure 1

Schematic mtDNA map illustrating the principle of fluorescent PCR analysis of rearranged mtDNAs. Under standard PCR conditions the sequences across rearrangement break-points are efficiently amplified as short products, whereas the equivalent products from wild-type mtDNA, many kilobases in length, are not.

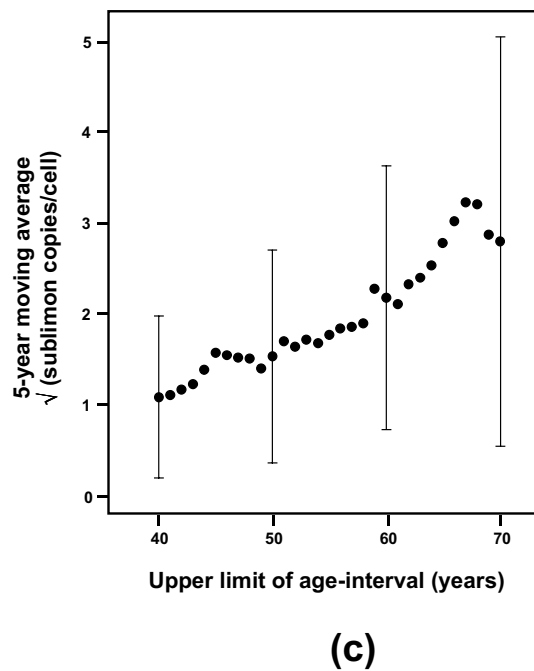
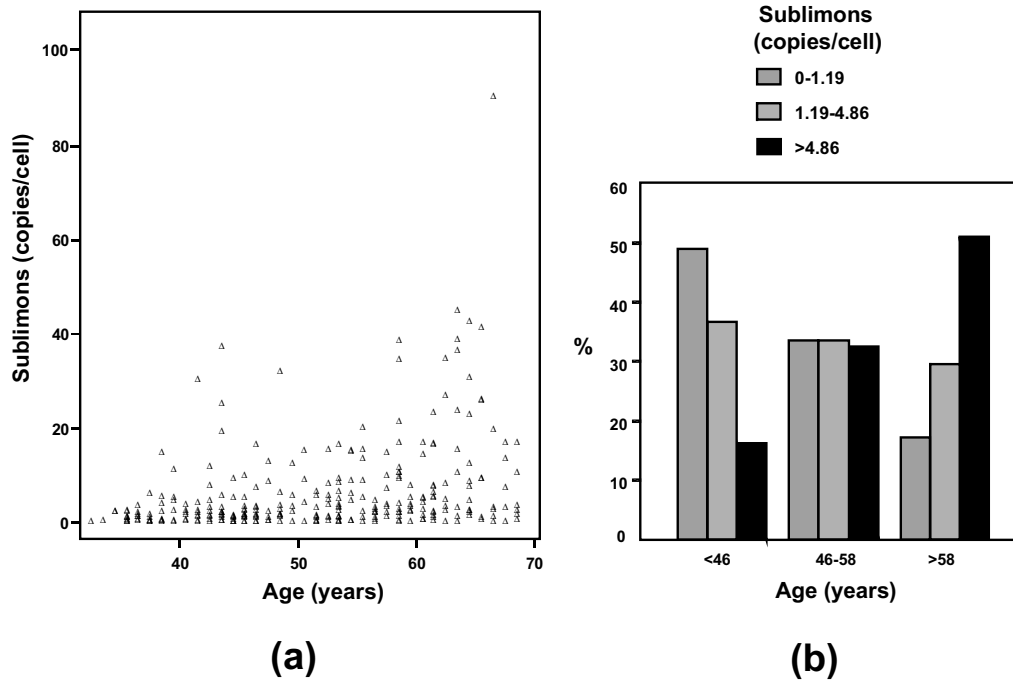


Figure 2

Relationship between sublimons and age, N=294. (a) Sublimon abundance (3.75 kb class) plotted against age. (b) Bar chart showing distribution of the men according to age and sublimon copy number, both divided into three equal sized groups by 33.3th and 66.7th percentiles of the continuous variables. Cases in each age group sum up to 100%. (c) The same data after square-root transformation and grouping into 5-year age intervals, plotted as a moving average (mean square-root of sublimon abundance, expressed as copies per cell, for all individuals within each 5-year age interval). For clarity, standard deviations are plotted only for the intervals ending at ages 40, 50, 60 and 70, but are similar for all intermediate intervals.

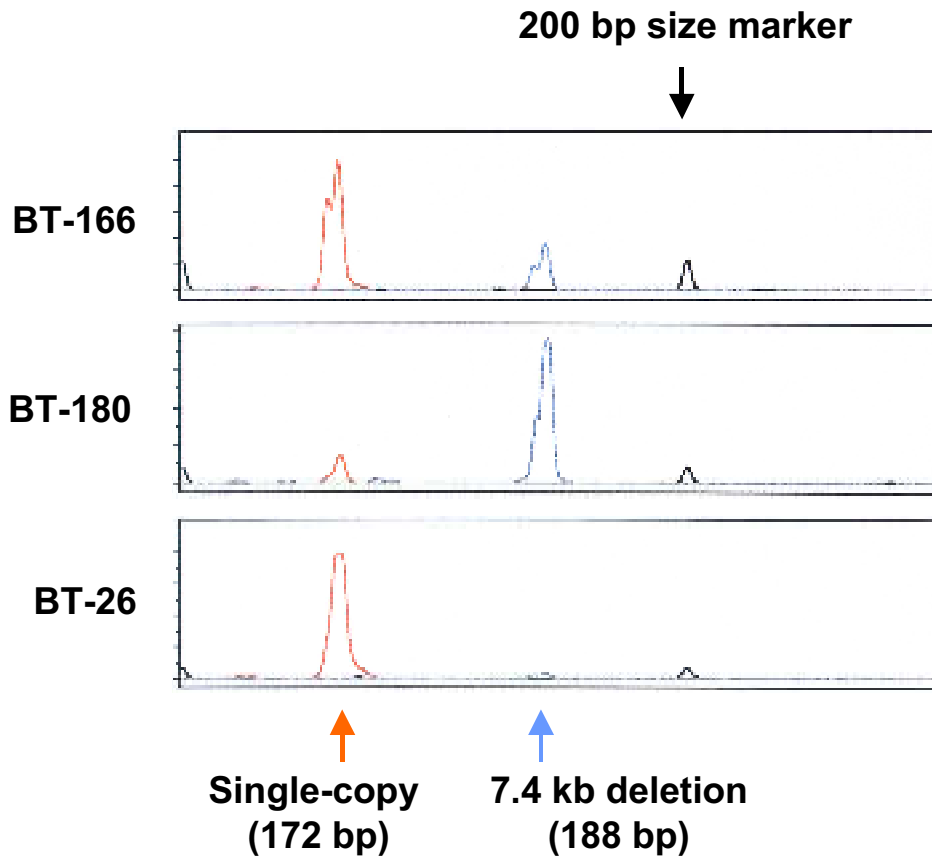


Figure 3
Semi-quantitative, multiplex fluorescent PCR analysis of the representation of the 7.4 kb deletion sublimon. Panels shown are outputs from GeneScan analysis carried out using the Applied Biosystems 310 capillary electrophoresis instrument, which resolves to the 1 nt level. The three panels shown represent examples of individuals with high (BT-180), intermediate (BT-166) and low (BT-26) abundance of the 7.4 kb deletion, compared with the single-copy internal standard.

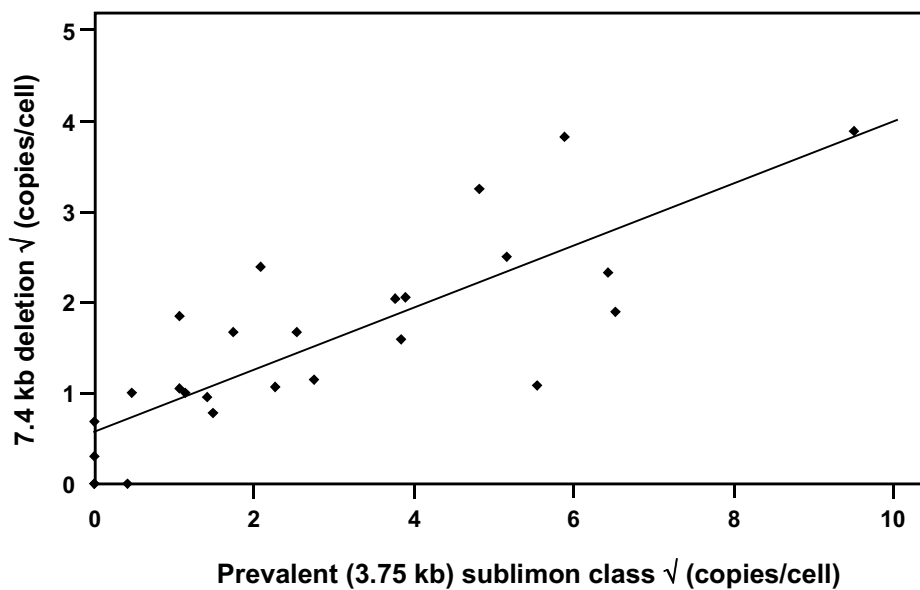


Figure 4
Correlation between the abundances of the two most prevalent myocardial sublimon classes. The abundance of the 7.4 kb deletion was measured in DNA from 26 individuals, and plotted against that of the most prevalent (3.75 kb) sublimon class in the same specimens.

ORIGINAL COMMUNICATIONS