Biochemical Composition of Coronary Arteries in Finnish Children

Seppo Yla-Herttuala, Tapio Nikkari, Jorma Hirvonen, Hannu Laaksonen, Matti Mottonen, Erkki Pesonen, Jyrki Raekallio, and Hans K. Åkerblom

To study the early features of atherosclerosis, we analyzed coronary artery intima medias from 63 boys and 30 girls who were newborn to 15 years old at the time of accidental death. Fatty streaks were found in five boys and one girl and, with one exception, were not present until the second decade of life. From birth there was a continuous increase in esterified cholesterol (EC) and a two- to threefold rise in free cholesterol and phospholipid. DNA, total protein, and collagen tended to rise after the first decade. The content of hyaluronic acid remained fairly constant, but sulphated glycosaminoglycans (GAGs) rose during the second decade; the highest increases were in dermatan and chondroitin sulphates. The content of arterial EC showed significant positive correlations with those of GAGs, but associations with the contents of DNA, total protein, and collagen were not significant. As judged by the observed gradual increase in arterial EC and a change in its fatty acid composition, there is a continuous increase with age in LDL-derived EC in the arterial wall. The mechanism of this accumulation is unknown, but the findings provide circumstantial evidence to support the concept that arterial GAGs may cause retention of plasma-derived lipids, which may subsequently be altered and internalized by the intimal cells.


To understand the pathogenesis of atherosclerosis, it is important to identify the biochemical changes associated with the development of the disease in the arterial wall. Animal models have provided valuable information in this regard, but direct projection of these data to humans is not possible.

Atherogenesis is a slow process that begins as early as in childhood and young adulthood.1 Fatty streaks appear in human aortas during the first, and in coronary arteries during the second decade of life; fibrous plaques follow about 10 to 20 years later.2,3 Several investigators have studied the biochemical composition of aortas in both children and adults,4-8 but to our knowledge there are only two reports9,10 on the biochemistry of coronary arteries in infants and children. This scarcity of studies is undoubtedly related to difficulties in sample collection and to analytic problems due to the small size of the vessels.

Finland has one of the highest incidences of coronary heart disease in the world.11 The total serum cholesterol levels in Finnish boys and girls are also higher than elsewhere.12,13 It is thus probable that the prevalence of early atherosclerotic lesions is high in Finnish children. The purpose of the present study was to analyze the composition of the coronary intima medias of 0- to 15-year-old Finnish children in order to identify the biochemical changes that precede and accompany the development of atherosclerotic lesions.

Methods

Collection of Specimens

The specimens were collected from medicolegal autopsies at the Universities of Helsinki, Kuopio, Oulu, and Turku, which are situated in the southern, eastern, northern, and southwestern parts of Finland, respectively. Children who had died violent deaths and had been autopsied between 1979 and 1983 were included in the study. In most cases, the bodies were cooled to 4°C as quickly as possible, usually within 2 to 4 hours after death. The mean postmortem time from death to autopsy was 3.4 days (range, 1 to 6 days) (Table 1). At the autopsy, the heart and aorta were removed and sent in an ice-cooled box by air (from Oulu or Kuopio) or by bus (from Helsinki) to the University of Turku, where they were further processed within 24 hours. The left main stem together with the left anterior branch (LAD), left circumflex branch (LC), and
Table 1. Age Distribution and Mean Postmortem Times of Autopsy in Children

<table>
<thead>
<tr>
<th>Age group (yrs)</th>
<th>Number of cases</th>
<th>Mean postmortem time (days ± so)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boys</td>
<td>Girls</td>
</tr>
<tr>
<td>Newborns</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0–&lt;3</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>3–&lt;6</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>6–&lt;9</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>9–&lt;12</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>12–&lt;15</td>
<td>11 (4)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>63 (5)</td>
<td>30 (1)</td>
</tr>
</tbody>
</table>

The numbers of coronary arteries with fatty streaks are given in parentheses.

right coronary artery (RC) were excised unopened from the heart and analyzed according to a protocol in which the LAD from the first heart, the LC from the second one, and the RC from the third one, etc., were used for the biochemical studies. The samples for the biochemical analyses were stored in tightly sealed plastic bags at -70°C until analyzed. The histologic and morphometric parts of the study involved a serial sectioning of the remaining coronary branches according to a similar protocol, followed by histochemistry and morphometry of the arteries. This report gives the results of the biochemical analyses.

The number of hearts collected was 106, and the number of hearts accepted for the biochemical analyses was 93 (age range, 0–15 years) (Table 1). Ten hearts were excluded because of transport delays, and three hearts because the child was older than 15 years. The deaths of the children were due to traffic accidents or other severe mechanical trauma (n = 65), drowning (n = 20), capital crime or suicide (n = 5), and burns (n = 3). Sudden infant deaths were not included.

**Dissection of Coronary Arteries**

Coronary arteries were freed from heart muscle, fat, and connective tissue; they were opened and washed with cold saline. After a visual estimation of the extent of the athero-sclerotic lesions, a sample was taken for histology, and the surface area of the vessels was measured with a special grid. Intima medias were carefully peeled off the adventitial layers with forceps under a dissecting microscope, were minced with a scalpel, and were weighed to obtain the wet weight. Since the intimal layer in the coronary arteries of young children is very thin and difficult to isolate, intima-medial samples were analyzed instead of intimas. Histologic controls showed that the samples contained the whole intima and 70% to 90% of the media. The level of the stripping plane was followed directly from coronaries large enough to meet the criteria for a constant dissection distance (see below), and indirectly from the rest of the samples by analyzing a random histologic sample of the remaining adventitial portion of the vessels. All specimens were dissected by the same investigator at 4°C. No macroscopic signs of autolysis were detected in any of the analyzed samples.

**Lipid Analyses**

The methods have been described in detail elsewhere. Briefly, lipids were extracted according to the method of Folch et al. and fractionated with thin-layer chromatography into esterified cholesterol (EC), triglycerides, free cholesterol (FC), and phospholipids (PL). The residue was vacuum-dried and weighed to obtain the tissue dry defatted weight, which varied from 0.33 to 14.10 mg (mean, 5.1 mg).

The EC fraction was saponified in 0.6 M KOH/ethanol, and the FC was recovered by extraction into petroleum ether. Cholesteryl ester fatty acids were extracted into petroleum ether after acidification of the saponification mixture and were esterified with 1.4 M HCl/methanol. The fatty acids were analyzed in a Hewlett-Packard 5880A gas chromatograph equipped with an OV-351 capillary column and a flame ionization detector. The results were expressed as percentages of the areas of all fatty acid peaks from 14:0 to 22:6.

The cholesterol was quantified as trimethylsilyl ether in another a Hewlett-Packard 5700A gas chromatograph by using a SE-30 capillary column and a flame ionization detector. Coprostanol was used as an internal standard. The total PL was determined as lipid-soluble phosphorus based on the assumption of an average phosphorus content of 4% for tissue PL.

The lipids from all samples collected were analyzed; however, fatty acids were not determined from newborns and four other cases because there was too small a sample size.

**Glycosaminoglycan, DNA, Collagen, and Total Protein Analysis**

An aliquot of dry, defatted tissue was digested with papain, and GAGs were precipitated by adding cetylpyridinium chloride (CPC), as described in detail earlier. The CPC-GAG precipitate was purified by precipitation with ethanol containing 2% potassium acetate. The GAGs were electrophoresed with two dilutions of standards by using a modification of the technique of Hata and Nagai on cellulose acetate membranes (Sepharose III, Gelman) in a Beckman Microzone electrophoresis chamber (0.1 M pyridine/0.47 M formic acid buffer, pH 3.1; 165 V, 25 minutes). The GAG fractions were stained with 1% Alcian blue and were cut off the membrane together with the blanks and the standards. The dye bound to each spot was eluted from the membrane and quantified by measuring its absorbance at 615 nm. The standard mixture contained hyaluronic acid (HA), dermatan sulphate (DS), and chondroitin sulphates A and C (CS A + C) (all from Sigma) and heparan sulphate (HS) isolated from pig aortic intima media.

DNA and total protein were both determined from the same aliquot of dry, defatted tissue by modifications of the techniques of Burton and Lowry et al., respectively; collagen was determined from another aliquot according to the method of Juva and Prockop. Because of the small size of the samples, GAG, DNA, collagen, and total protein analyses were not made from all coronary arteries. According to the authors’ experience, at least 3.5 mg of dry, defatted arterial tissue is needed for GAG analysis and 2.0 mg for DNA and total protein determination. GAG analysis was always given the priority, and DNA, total protein, and collagen were only measured if there was enough sample left.

The effect of postmortem time on the biochemical composition of coronary arteries has been studied previously by keeping coronary arteries from swine at 4°C for periods up to 7 days. There were no alterations in the composition of substances other than sulphated GAGs, which declined during the first 4 to 5 postmortem days. It is thus likely that the values for sulphated GAGs are underesti-
mated in all postmortem materials. In the present study, the mean postmortem times of the age groups did not differ (Table 1), and the results were considered reliable enough for the present purposes.

**Histological Procedures**

A tissue sample was taken for formalin fixation from a constant, distal position of the vessels if the arteries were long enough to allow the following dissection distance: 2.5 cm distal to the origin of LAD, 2.0 cm distal to the origin of LC, and 3.0 cm distal to the origin of RC. The sections were stained with hematoxylin/eosin and Verhoeff/Van Gieson, and the relative thickness of intima to media was measured from a magnified image of the samples at 10 random sites along the vessel wall circumference.

**Calculation of the Results and Statistical Methods**

The results were calculated both per unit surface area (i.e., content) and per unit dry defatted tissue weight (i.e., concentration). Linear correlations were calculated from individual observations. For presentation, the age groups were formed at 3-year intervals as shown in Table 1, and the individual or combined groups were used for calculations of statistical significances by t tests. Except in Table 3, coronaries with fatty streaks were included in the means.

**Morphologic Findings**

The youngest subject whose coronaries showed a fatty streak was a 7.9-year-old boy, although generally, fatty streaks appeared in the coronary arteries only after age 12, more often in boys than in girls (Table 1). All lesions were single, were located in proximal parts of the coronary branches, and covered 0.2% to 2.0% of the surface area.

The relative intimal thickness increased significantly with age (Figure 1). The steepest rise occurred during the first decade, whereas no changes were found in collagen, total protein, and total DNA (means, 12.7 and 7.8 µg/cm², respectively, NS) (data not shown).

No gender differences were found in any of these non-lipid constituents, although boys in the oldest age group tended to have more DS in their coronary arteries than girls (means, 12.7 and 7.8 µg/cm², respectively, NS) (data not shown).

**Lipids**

The contents of both FC and PL showed a steady, two- to threefold increase with age (r = 0.618; p < 0.001, and r = 0.540; p < 0.001, respectively; Figure 4 A). In accordance with earlier reports, 4, 7, 9 results calculated per dry

![Figure 1](image1.png)

**Figure 1.** The ratio of the thickness of the intima to that of the media (mean ± SEM) in different age groups. The numbers of specimens in each age group were 5, 8, 11, and 13. Correlation between age and the intima/media ratio based on individual observations was r = 0.411, p < 0.05.

![Figure 2](image2.png)

**Figure 2.** The contents (A) and the concentrations (B) of total GAG (•), total protein (○), DNA (■), and collagen (□) in the coronary arteries as a function of age (mean ± SEM). The numbers of specimens in each age group analyzed for total GAG were 9, 13, 15, and 14. The respective numbers analyzed for both total protein and DNA were 5, 8, 11, and 13, and those for collagen 0, 3, 7, and 7, *p < 0.05 in comparison to the mean of children younger than 12 years, by Student's t test. d.t. = dry defatted tissue.

![Figure 3](image3.png)

**Figure 3.** The contents (A) and the concentrations (B) of GAG fractions in the coronary arteries in various age groups (mean ± SEM). The numbers of specimens in the age groups were 9, 13, 15, and 14, respectively. *p < 0.05 in comparison to the mean of children younger than 12 years, by t test. HA = hyaluronic acid; DS = dermatan sulphate; CS A + C = chondroitin sulphates A and C; and HS = heparan sulphate; d.t. = dry defatted tissue.
defatted tissue were high in newborns (Figure 4 B) obviously because of inaccuracy in weighing the disrupted tissue samples after lipid extraction. In the older age groups, the concentrations of both FC and PL rose with age ($r = 0.422; p < 0.001$; and $r = 0.460; p < 0.001$, respectively). There were no significant differences between genders in any of the age groups (data not shown).

The EC content increased continuously from birth to the age of 15 years ($r = 0.557; p < 0.001$; Figure 5 A). The results were essentially the same when they were expressed in terms of concentration ($r = 0.514; p < 0.001$), although the values for newborns appeared to be higher than the respective contents (Figure 5 B). No significant gender differences were observed (data not shown).

The percentage composition of cholesteryl ester fatty acids in the youngest children was characterized by a large proportion of saturated fatty acids, especially palmitate and stearate, whereas the relative amounts of linoleate and arachidonate were low (Figure 6 A). With age, significant increases took place in the percentages of cholesteryl ester linoleate ($r = 0.470; p < 0.001$) and arachidonate ($r = 0.513; p < 0.001$) in both sexes, with a simultaneous decrease in stearate ($r = -0.391; p < 0.001$) and palmitate ($r = -0.493; p < 0.001$).

The relative content of cholesteryl oleate, frequently used as a marker of the intracellular cholesteryl ester synthesis, remained fairly constant during the first decade, suggesting that the majority of cholesteryl esters were not synthesized in the arterial wall. In fact, with age the mean cholesteryl ester fatty acid composition appeared to approach that of serum low density lipoprotein (LDL) (Figure 6 B).

### Table 2. Correlations of the Contents and Concentrations of Coronary Glycosaminoglycans, Total Protein, DNA, and Collagen with Those of Esterified Cholesterol and with Age in Children under 12 Years

<table>
<thead>
<tr>
<th></th>
<th>Hyaluronic acid</th>
<th>Heparan sulphate</th>
<th>Dermatan sulphate A and C</th>
<th>Total GAGs</th>
<th>Total protein</th>
<th>DNA</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Content ($\mu g/cm^2$)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC content</td>
<td>$0.420^*$</td>
<td>$0.522^+$</td>
<td>$0.472^+$</td>
<td>$0.412^*$</td>
<td>$0.562^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>$-0.155$</td>
<td>$0.152$</td>
<td>$0.014$</td>
<td>$0.170$</td>
<td>$0.110$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                     |                  |                  |                           |            |              |     |         |
| **Concentration (mg/100 mg d.t.)** |                 |                  |                           |            |              |     |         |
| EC concentration    | $0.230$          | $0.226$          | $0.253$                   | $0.174$    | $0.297$      | $0.262$ | $0.067$ |
| Age (yrs)           | $-0.323$         | $-0.051$         | $0.107$                   | $0.113$    | $-0.053$     | $-0.064$ | $-0.428^*$ |

$n = 37$, except for total protein and DNA, $n = 24$, and for collagen, $n = 10$. d.t. = dry defatted tissue.

*p < 0.05; ^p < 0.01; ^p < 0.001.
the results were expressed in terms of concentration, correlations between EC and GAGs did not reach the level of statistical significance. The correlations of arterial EC with the contents or the concentrations of DNA, total protein, or collagen were insignificant.

**Comparison of Lesion-Free Vessels to Those with Fatty Streaks**

When the specimens in the oldest age group were divided according to the presence or absence of fatty streaks, significantly higher contents of FC, EC, PL, and DS were present in the affected coronaries. Corresponding results based on the concentrations only showed an increase of EC (Table 3). The results may indicate a general increase in the thickness of the affected arteries. However, no difference was detected in the intima/media ratio, which was probably due to the distal origin of the histologic samples.

**Discussion**

The interpretation of results obtained from human autopsy material is always endangered by the possibility of inherent bias in the selection of autopsied cases. This was minimized in the present work by including only successive, accidental deaths.

In most earlier studies, the results have been expressed per unit of dry and/or defatted tissue weight, which actually gives a percentage value of the component of the total material. This way of reporting the results is without problems because the percentage of a component is dependent on simultaneous changes in the amounts of other components. Thus, an increase in any component that does not keep pace with the increase of the bulk of the material is reflected as a decrease of that component. This is especially true in adults, where an exponential accumulation of calcium salts in severely affected vessels significantly alters the relative proportion of the arterial nonlipid material. In newborns, on the other hand, the sample sizes are very small (0.3 to 0.9 mg of dry defatted tissue), and the results calculated per unit of dry defatted tissue appear to be undercorrected because of difficulties in accurately weighing the disrupted tissue samples after lipid extraction (Figures 4 B and 5 B).

In the present study, the results were calculated both in relation to dry defatted tissue (concentration) and per vessel surface area (content). The latter method has the advantage of reflecting the overall situation in the isolated intima media, but suffers the drawback of not correcting the results for intima-medial thickness. The dissection plane between media and adventitia was controlled histologically and was fairly constant: the isolated samples contained the whole intima and 70% to 90% of the media with no adventitial contamination. The contents thus give estimates of the absolute amounts of the components in the arterial wall. An additional way of expressing the results would be on a volume basis, but this was not feasible in the present study.

The relative intimal thickness increased with age (Figure 1). Nevertheless, during the first decade, there were no rises in the contents or in the concentrations of intima-medial collagen, total protein, and GAGs, and, as in human aortas, there was a fall in DNA during the first years of life (Figure 2). That the thickening of the intima did not clearly show in the chemical measurements was probably due to the fact that the analyses were made from intima media, and the relatively stable medial layer could shadow small changes taking place in the intima. During the second decade of life, the contents of all these components started to increase, but no clear rises were observed in their concentrations (Figure 2).
The lipid composition in newborn intima medias resembled that of cellular membranes by having a FC/PL ratio of 0.72, which rose to 0.90 at the age of 2 to 3 years (Figure 4). The parallel rises in FC and PL during the first decade, when their molar ratio remained fairly constant (0.90 to 0.94), obviously reflect the growth of the arteries. 7

From birth onward, a continuous, age-related increase was observed in intima-medial EC (Figure 5). This agrees with earlier reports from human aortic intima9 and newborn media. 4,8 while studies on human coronary arteries are conflicting. Scott et al. 9 found only negligible amounts of EC in the coronary intima medias of children under 10 years of age, whereas the EC concentrations reported by Meyer et al. 19 in whole coronary arteries from the same age group were appreciably higher than ours and were not age-dependent. The discrepancies are probably due to different analytical methods. The age-related increases in coronary cholesteryl ester linolate and arachidonate, with the concomitant decrease in saturated fatty acids, suggest that arterial cholesteryl esters are derived from the circulation in that their fatty acid composition approaches that of serum LDL with age (Figure 6). These results agree with those from studies on human aortic tissue, 3-7 and the presence of LDL in aortic intima has been confirmed both by immunohistology 28'29 and by immunoelectrophoretic techniques. 5,29

There are only a few reports on the amounts of GAGs in human coronary arteries. 30-32 Children’s coronary arteries appear to have higher percentages of HS (47%) and lower percentages of CS A + C (27%) and DS (14%) than those of adults (32%, 34%, and 24%, respectively, see reference 32). The rises in sulphated GAGs during the second decade of life are parallel to those occurring with age in adult coronary arteries. 32

The contents of arterial GAGs correlated significantly with the content of EC even after the confounding effect of age was eliminated, whereas no correlations were observed between the respective concentrations (Table 2). This discrepancy may be related to the different nature of the expressions used, but it may also reflect a variation in sample thickness, i.e., dissection plane. If the relationship between EC and GAGs is solely caused by variation in sample thickness, EC could be expected to correlate also with DNA and total protein contents, which are even more evenly distributed throughout the entire intima media than are GAGs. However, Table 2 shows that these associations were clearly weaker than those between EC and GAGs. GAGs might thus reflect the volume of the aqueous extracellular space available for LDL after its entry into the intima. LDL may be retained in this space through molecular sieving and GAG (proteoglycan)-mediated complex formation. 33-38 Accordingly, immunofluorescence studies have revealed a close topographic relationship between apolipoprotein B (LDL) and GAGs in the arterial wall. 28 Whether this process predisposes arteries to atherogenesis is not known, but it has been recently shown that proteoglycan-LDL complexes can lead to cholesteryl ester accumulation in macrophages in vitro. (Ylä-Herttuala S, Jaakkola O, Solakivi-Jaakkola T, Kuivalaniemi H, Nikkari T. The effect of proteoglycans, collagen and lysyl oxidase on the metabolism of low density lipoprotein by macrophages; unpublished data). 37,38

Acknowledgments

The authors thank Marja Joussimies, Ramona Ratalahti, Seija Koivuniemi, Ulla Saarijoki, and Päivi Koivumäki for skillful technical assistance and Eija Kyrölä for manuscript preparation.

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Index Terms: atherosclerosis • children • coronary arteries • fatty acids • glycosaminoglycans • lipids
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doi: 10.1161/01.ATV.6.2.230

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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