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Dual role of infections as risk factors for coronary heart disease.

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Abstract

Aims. The aim of the study was to explore whether exposure to microbial agents determines the prevalence of acute coronary events.

Methods and results. Patients with unstable angina pectoris and myocardial infarction (N=335) and their paired controls were investigated. The subjects answered a questionnaire about their childhood contagious diseases: varicella, scarlet fever, measles, rubella, mononucleosis and mumps. Blood samples were taken for bacterial and viral serology. The odds ratio for CHD was highest in the upper quartile of the enterovirus (EV), herpes simplex virus (HSV) and *Chlamydia pneumoniae* HSP 60 IgG antibody titers (1.86, p=0.001, 1.57, p<0.048 and 1.70, p=0.016 respectively). The antibody titers increased cumulatively the risk for CHD (odds ratios 1.89, 2.24, 3.92 and p-values <0.001, 0.001 and 0.047). Childhood contagious diseases (n=6) had a protecting effect against CHD (odds ratio 0.86, p=0.013). The risk for acute coronary events decreased significantly with increasing number of childhood contagious diseases (p=0.007).

Conclusions. Infections have a dual role in the genesis of CHD. EV, HSV and *Chlamydia pneumoniae* heat shock protein 60 IgG antibodies are associated with increased risk for CHD. Protection from infections usually suffered during the childhood before the era of MMR vaccination may predispose the individual to CHD.

Keywords

Enterovirus – herpes simplex virus - *Chlamydia pneumoniae* heat shock protein 60 - infection burden – hygiene hypothesis

Introduction

Coronary heart disease (CHD) is considered as an inflammatory disease. There is evidence that the disease might have its roots already in childhood [1]. Traditional risk factors such as smoking, diabetes, hypercholesterolemia and hypertension are not sufficient to explain the high incidence of CHD in the western world [2,3]. Infections might play etiological role in CHD. However, exposure to infections in childhood might be necessary for development of normal immunological defense mechanisms as suggested in hygiene hypothesis.

There is earlier evidence that chronic or repeated enterovirus (EV) infections are associated with increased risk for myocardial infarction. High titers against EV common antigen in sera of men without evidence of heart disease taken years before the coronary attack have been reported in two large population studies [4,5] but not confirmed in a third study of dyslipidemic men [6]. The time span between antibody measurements and the actual event was years.

The aim of the present study was to explore whether infections starting from childhood determine the prevalence of acute coronary syndromes.

Patients and methods

The study population consisted of 351 patients, 276 men and 75 women, admitted for acute myocardial infarction (AMI) or unstable angina pectoris to the heart intensive care unit, Lund University Hospital between March 1999 and April 2002.

Inclusion criteria for patients were age < 80 years, no signs of cognitive intellectual disability as well as competence to apply to the research protocol. Of these patients 21 died before they could be interviewed. Of the invited patients 48 chose not to participate. After admission 12 patients were excluded from the study with following diagnoses: unspecified precordial pain in seven patients, atrial fibrillation, pericarditis, myocarditis, pulmonary embolism and aortic aneurysm. AMI was diagnosed in 238 patients. Of those 113 had a Q-wave infarction and 125 non-Q-wave infarction. Unstable angina pectoris was diagnosed in 113 patients. An earlier MI had been diagnosed in 37 patients.

Control individuals were selected using the population register. There were 355 controls. They were chosen from the same area, usually from the same blocks and were matched with age \pm 2 years, sex, and parish. In almost every case the control lived in the same block as the patient. Inclusion criteria were: no history of definite or suspected coronary heart disease or stroke, no operations or chemotherapy within the previous 4 weeks. A

positive history of angina was deemed to be present if chest pain in any location was related to exercise and relieved by rest.

A research nurse made a visit to the control person within 5 days. The controls received written information about the study and signed consent to participate. A questionnaire was filled in and 70 ml of blood was collected. The blood vials were put on ice and transferred to the central laboratory for centrifugation.

The participants completed questionnaires on general background characteristics and demographic data including history of infections usually suffered during the childhood before the era of MMR vaccination, smoking, marital status, socio-economic data and the medical history of the family.

AMI was diagnosed if two of the following criteria were fulfilled: ST-elevations followed by T-wave inversion or new Q-waves in the ECG, typical chest-pain with a duration of >20 min, an increase of CKMB to more than twice the upper limit of the normal value. Unstable angina was diagnosed in patients with continuous ischemic chest pain and transient or persistent ST-segment depression (>1mm) or T-Wave inversion (>1mm) and/or elevation of CKMB (>10)/Troponin T (>0.10).

Blood samples for TnT and CK-MB samples were obtained on admission to the hospital and at 10 and 20 hours. Study samples from the patients were collected within 24 h after the diagnosis. Blood was collected for future use, seven tubes of serum without additives, and 3 tubes of EDTA plasma and one tube of citrate

plasma. The tubes were immediately placed in a refrigerator to be centrifuged 3,200 rpm for 10 minutes; next the plasmas were separated and immediately frozen and stored in aliquots at -70. “Buffy coat” fraction of the centrifugate was saved from 202 patient and controls. Serum total cholesterol and LDL-cholesterol concentrations were measured.

Bacterial serology included *C. pneumoniae* and *C. pneumoniae* heat shock protein 60 (*C. pneumoniae* HSP60) IgA and IgG as well as *H. pylori* IgA and IgG antibody titers. Viral serology included determination of cytomegalovirus, EV and herpes simplex virus (HSV) antibody titers.

C. pneumoniae antibodies were detected by microimmunofluorescence (MIF). Antigen was produced from type strains of *C. pneumoniae*, *C. psittaci* and *C. trachomatis* grown in embryonated eggs. Specific antibody reactivity to *C. pneumoniae* was determined. Antibody titers were obtained as reciprocal dilution steps.

IgG and IgA antibodies against the *C. pneumoniae* HSP60 were measured with EIA as described in detail elsewhere [7]. Briefly, microtiter plates were coated with a recombinant *C. pneumoniae* HSP60 protein produced in *Bacillus subtilis* [8] at a concentration of 5 µg/ml in PBS (pH 7.4) overnight at 37°C. After coating, the plates were incubated for 2 h at 37°C with duplicate samples diluted 1:50 for serum IgA and 1:200 for serum IgG. The plates were then incubated for 2 h at 37°C with alkaline phosphatase-

conjugated anti-human IgA (Caltag Laboratories, Burlingame, Calif., USA) and anti-human IgG (Sigma, St. Louis, Mo., USA). Following a 30-min incubation at 37°C with a substrate solution containing 1 mg of p-nitrophenyl phosphate disodium in 1 ml of carbonate MgCl₂ buffer, absorbance was measured at 405 nm. The results were expressed as EIA units (EIU) by multiplying the optical densities by 100.

IgG and IgA antibodies to *Helicobacter pylori* (*H. pylori*) were analyzed from serum with an in-house enzyme immunoassay. The antigen used was an acid glycine extract from *H. pylori* strain NCTC 11637 [9]. The lower limits for the raised antibody titers were 700 for IgG and 70 for IgA antibodies.

Antibodies to cytomegalovirus (CMV), HSV and EV were measured by enzyme immunoassay (EIA). Strains of CMV (AD169), HSV (a local HSV-1 isolate) and EV (Coxsackie B2) were grown in appropriate cell cultures. The cultures were harvested when an extensive cytopathogenic effect was noted. Antigen was prepared after differential centrifugation and sonication. Positive and negative antigens were used for CMV and HSV. The coxsackie B2 antigen produced has broad reactivity and will detect an antibody response to different types of enterovirus. Positive and negative control sera were included in all test runs. Positive and negative control sera were included in all test runs. Results were expressed as the quotient between the observed optical density (OD value) in the test and the cut-off for a positive

result. A doubling of the quotient implies a titre change of half a 10 log step and a quadrupling one log step a tenfold increase in titre.

The Lund University Hospital ethical committee approved the study. A written informed consent for participation in the study was obtained from all subjects.

Statistical methods

The group means of continuous variables were compared using one-way ANOVA. Chi-square test was used for categorical variables. The effect of viral infections on the risk of CHD was analyzed with logistic regression models. The goodness of fit of the models were evaluated using Hosmer-Lemeshow test. The results are expressed as odds ratios (OR), 95% confidence intervals and P values. Wald's test and trend test were used to evaluate the statistical significance of the coefficient of logistic models. A ratio of less than 1.0 indicates a beneficial effect, and is significant if the confidence interval's upper value is below 1.0. Two-sided p-values <0.05 were considered statistically significant. The data were analyzed using SPSS for windows software version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

The mean age of the patients was 63 years (32-82 years, median 64 years), 20% being 55 or younger and 30 % 70 or older. AMI

was diagnosed in 238 patients and 113 had unstable angina pectoris. Serum total cholesterol was in controls 5.76 mmol/l and in patients (after infarction 4.22 mmol/l). LDL cholesterol values were 3.67 mmol/l and 2.41 mmol/l. An earlier AMI had been diagnosed in 49 of the 335 patients (15%). Patients had in 25 % and controls in 15% first-degree relatives with heart disease before 65 years of age ($p < 0.001$). Patients and controls smoked equally often, in 9 and 11 %.

Infections during the childhood

The childhood contagious diseases (CCD) before the era of MMR vaccination were more frequent in the controls than in the patients. P-values for the difference between the controls and the patients was 0.032 for varicella, 0.054 for scarlet fever and 0.063 for measles (Table 1). The number of suffered CCD was related to the risk of a coronary event in a way that the odds ratio decreased with increasing number (from one to six) of CCD. The decrease of the risk ranged from 0.65 to 0.11 (Table 2). One additional infection had a protective effect of 14.3 % (odds ratio 0.857, $p = 0.007$ for the trend).

Viral and bacterial antibody titers

The microbial antibody titers in controls and patients are presented in the table 3. Ex-smokers had higher antibody titers for C.

pneumoniae IgG and IgA ($p=0.012$ and 0.002) but there was no correlation between any microbial antibody titers on hypercholesterolemia or marital status (married/unmarried). EV antibody titers had a trend to be higher in patients having had an earlier myocardial infarction than in other patients (1.59 and 1.53, $p=0.102$). Microbial titers were similar in the subjects whether or not the first-degree relatives had an infarction before getting 65 years.

Age and sex adjusted EV and HSV antibody titers were significantly higher in the patients than in the controls ($p<0.001$ and 0.038). Odds ratio in the second, third and fourth EV antibody titer quartile was 0.98, 1.81 and 1.86. The significance of the difference between the fourth as compared to the first quartile was 0.005. The corresponding p-value comparing HSV antibody titer quartiles was 0.048. Also in highest *C. pneumoniae* HSP60 IgG titer quartile was the odds ratio significant (Table 5).

Infection burden

EV, HSV and *C. pneumoniae* HSP60 IgG antibody titers increased cumulatively the risk for coronary event. The cumulative odds ratios were 1.89, 2.24 and 3.92 correspondingly (Table 4). The odds ratio was highest in the upper quartile of both the EV and HV antibody titers 2.16 (CI95%: 1.36-3.43) and 1.41 (CI95%: 0.82-2.18, $p=0.149$) respectively (Table 6). The number of childhood

infections (N=6) showed a negative correlation to coronary events the odds ratio being 0.89 (CI95%: 0.79-0.99, p=0.045).

Discussion

EV titers were high whether the patients had had an earlier myocardial infarction or not. Actually there was a trend of the titers being higher in those with earlier infarction which is in contrast to the study of Reunanen et al [5]. The risk for coronary event was associated with high EV, HV and *C. pneumoniae* HSP60 titers and was even more pronounced if the antibody titers were high against multiple agents supporting the infection burden hypothesis. On the other hand the risk for acute coronary events decreased with increasing number of childhood contagious diseases.

Risk for CHD

Since the original studies of Saikku et al published in 1988 *C. pneumoniae* bacteria have been considered as the main vascular pathogens increasing the risk for CHD [10]. Also in our study *C. pneumoniae* HSP60 IgG antibody titers were elevated. The relative risk for MI seems to be increased during acute infections in general [11-13]. The observed antibody responses for microbes in our study may reflect acute but also reactivated latent or chronic infections. Their effect seems not to be mediated by the known risk factors such as hypertension and diabetes [5].

It may well be, as earlier suggested, that a process that eventually leads to CHD is initiated in early life by multiple infections acquired in the childhood [1,14]. Infections and especially viral infections have been reported in children to associate with thickening of the intima of the coronary arteries [14] and thickening of the carotid arteries [15].

Hygiene Hypothesis

Stimuli by infectious diseases or vaccinations during early life might be necessary for normal development of the immune response and to protect from CHD as earlier suggested for the development of allergic diseases and multiple sclerosis.

The occurrence of childhood contagious diseases showed a negative correlation to acute coronary syndromes. Exposure or re-exposure to infections can influence the developing immune system and might be necessary for normal development of immunological defense mechanisms.

Certain allergic diseases might also be prevented by exposure to infectious agents during early childhood. Measles infection seemed to protect against the development of atopy in African children [16] even if this could not be corroborated in further studies [17]. According to an explorative analysis of the literature the presence of indirect markers of infection associated with 20% protection for atopic dermatitis, 30% for allergic rhinitis and 40% for asthma [18]. Interestingly, allergic disorders have been

reported to be associated with the development and progression of atherosclerosis [19].

A high contact exposure within the first 6 years of life with younger siblings less than two years of age in the same household was recently shown to be associated with a reduced risk and delayed onset of multiple sclerosis [20]. The number of siblings was used as a marker of infection load during early life. Higher infant contact in early life was associated with reduced EBV antibody titers and a reduced likelihood of infectious mononucleosis among healthy controls.

In contrast to childhood infections such as measles common viral respiratory infections in early childhood are reported not to confer protection from subsequent identical infections [21]. According to our study “the hygiene hypothesis” [18, 20] might also be relevant for explaining development of CHD. Improved hygiene in the early childhood might partially explain the rise of the greatest epidemic of the 20th century, the coronary heart disease.

Healthy controls

In this study the controls were matched with respect to age, sex, living area, parish, season and epidemiological status in the community. The controls did not have a history of definite or suspected coronary heart disease or stroke. The fact that cases and controls in our study were neighbours adjusts for the socioeconomic status; one tends to live nearby people from one's

own social class. Seasonal variation and epidemiological situation through their influence on the antibody titres do not affect the results because blood samples for antibody determinations from control subjects were taken within 5 days of the coronary event and blood collection from the matching patients. Smoking status was similar in cases and controls but family history of early CHD was more often positive in the patients.

Mechanism of infection initiated CHD

Infectious pathogens initiate endothelial damage [22,24]. Acute infections worsen the endothelial cell function which might not have returned quite normal even after one year [25]. Endothelial cell function of diabetic children gets still worse after acute infections [24].

Functional/morphological damage during an acute infection is reflected as a decreased availability of NO, which limits the ability of the coronary arteries to dilate. This mechanism might be mediated by cytokines such as interleukin-6 [26] and tumor necrosis factor α [27,28]. Our recent experimental studies have shown a profound and diffuse impairment of endothelial function of coronary vascular bed during acute *C. pneumoniae* infection in piglets [23]. A brief exposure of veins to endotoxin has been shown to initiate endothelial stunning for 48 h [29]. The impaired dilatory function of coronary vessels might end up nearly a loss of dilatory mechanisms underlying coronary flow regulation. These

mechanisms might be important especially in patients with unstable AP.

It is unclear whether elevated antibody titers to EV reflect an older, already passed infection or maybe a new or reactivated acute infection. In earlier studies EV [4,6] and HSV antibodies [6] were considered as a sign of frequent infections. EV and HSV exist latent in the tissues. These latent infections could be activated after the primary infection by a superinfection with another agent [30]. Differences in host inflammatory response to infection comprise a potentially important factor modifying the inflammatory response of the host [31].

Pathogen burden

In this study three pathogens EV, HSV and *C. pneumoniae* had a positive correlation to the incidence of CHD. Pathogen burden with many infectious agents has been reported to increase additionally the risk of cardiovascular events [32,33,34]. A recent study found a positive correlation between the number of pathogens in CHD patients undergoing angiography and the impairment of coronary artery reactivity to acetylcholine, an endothelium-dependent vasodilator [35]. Our study supports the pathogen burden hypothesis.

Limitations of the study

The traditional risk factors such as hypertension, diabetes and hypercholesterolemia might have influence on the incidence of infections. However, in an earlier study there was no correlation of hypertension and diabetes on microbial titers [5]. This could be shown also in the patients in this study.

The frequency, duration and intensity of infections vary. The strength, the quantitative effect of infections could not be determined. The methods could not differentiate serological “scars” from responses due to recent infections. Subclinical infections may have remained undiagnosed even if they might have influenced on the pathogenesis of CHD. The final atherogenic potential of infections is dependent on combined influences of genetic [36], toxic and protecting factors.

Conclusions

Our study supports the idea that EV, HSV and *C. pneumoniae* infections are pathogenetic factors for CHD and their pathogenic burden still increases the risk for CHD. Aberrant immune response according to the hygiene hypothesis might be a contributory factor. However, the evidence is still fragmentary.

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Conflict of Interest: none declared

Table 1. Protective effect for coronary event of the six ordinary contagious diseases usually suffered during the childhood before the MMR vaccination era. Age and sex adjusted odds ratios of infections for a coronary event. Logistic regression analysis.

Infection.	Number c/pt*	%positive in c/pt*	Odds ratio	p-value
varicella	258/216	79/72	0.668	0.032
scarlet fever	88/62	27/21	0.693	0.054
measles	267/225	81/75	0.698	0.063
mumps	233/199	72/66	0.755	0.105
mononucleosis	14/9	4/3	0.670	0.361
rubella	185/165	57/55	0.914	0.581

*c/pt=controls/patients

Table 2. Age and sex adjusted odds ratios adjusted for a coronary event of increasing number of infections usually suffered during the childhood before the MMR vaccination era. One additional infection has a protective effect of 14.3 % (odds ratio 0.857, $p=0.007$ for the trend).

Number of infections	Subject number c/pt*	Odds ratio	p-value
0 (reference)	13/23	1	
1	33/38	0.653	0.311
2	43/45	0.596	0.204
3	73/68	0.527	0.098
4	113/92	0.459	0.038
5	44/34	0.458	0.044
6	5/1	0.112	0.057

*c/pt=controls/patients (n=324/301)

Table 3. Microbial immunofluorescence titers in the study population. EV, HSV and CMV results are expressed as an index for sample optical density/cut-off optical density. *C.pneumoniae* IgG and IgA titers present reciprocal dilution steps. *C.pneumoniae* Hsp60 igG and IgA antibodies are in arbitrary EIA units. *H. pylori* titers are expressed as % of raised titers.

Group	N	EV*	HSV	CMV	Cpn		CpnHsp60		H. pylori	
					IgA	IgG	IgA	IgG	IgA	IgG
Controls	335	1.46	3.82	3.14	3.35	6.46	3.74	0.32	28	40
Patients	351	155	4.06	3.23	3.91	6.60	4.11	0.39	26	39
Angina	113	1.59	4.10	3.18	4.21	6.88	0.40	0.41	25	38
AMI	238	1.53	4.03	3.28	3.73	6.46	0.42	0.38	26	38

Cpn= *C.pneumoniae*, Angina = angina pectoris, * p<0.0001

Table 4.

A. Odds ratios adjusted for age and sex in quartiles of antibody titers for the risk of an acute coronary event. Reference category is I (logistic regression analysis). HSP60 = heat shock protein 60.

Titer	I	II	III	IV	p-value
Enterovirus	1.00	0.977	1.813	1.864	0.005
Herpes simplex virus	1.00	0.705	0.841	1.565	0.048
<i>C. pneumoniae</i>	1.00	1.390	0.988	1.701	0.016
HSP60 IgG					

Table 5. Infection burden for the risk of an acute coronary event i.e. odds ratios and 95% confidence intervals for enterovirus, herpes simplex virus and *Chlamydia pneumoniae* heat shock protein 60 immunoglobulin G.

Separate logistic models.

Variable	Odds ratio and confidence intervals
Enterovirus	1.89 (CI: 1.34-2.66, p<0.001)
Herpes simplex virus	2,24 (CI: 1.39-3.62, p=0.001)
<i>C. pneumoniae</i>	3.92 (CI: 1.02-15.12, p=0.047)
HSP60 IgG	

Table 6.

Odds ratios and 95% confidence intervals for viral antibody titers and self-reported childhood diseases on the risk of acute coronary event. Reference category is quartile I (logistic regression analysis).

Variable	Odds ratio and confidence interval
Age	0.99 (CI95%: 0.98-1.01, p=0.472)
Sex	1.07 (CI95%: 0.71-1.61, p=0.736)
Childhood infections (0-6)	0.89 (CI95%: 0.79-0.99, p=0.045)
Enterovirus	P<0.001
Quartile I	1.000
Quartile I	1.08 (CI95%: 0.68-1.72, p=0.756)
Quartile II	1.93 (CI95%: 1.21-3.01, p=0.005)
Quartile IV	2.16 (CI95%: 1.36-3.43, p=0.001)
Herpes simplex virus	p=0.038
Quartile I	1.000
Quartile I	0.74 (CI95%: 0.46-1.17, p=0.196)
Quartile II	0.82 (CI95%: 0.52-1.30, p=0.398)
Quartile IV	1.41 (CI95%: 0.88-2.25, p=0.149)

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