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PATHOBIOLOGICAL DETERMINANTS OF ATHEROSCLEROSIS IN YOUTH (PBDAY STUDY)

DRAFT PROTOCOL

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1. INTRODUCTION

The present protocol describes the general outlines of a study which, in continuation of the International Atherosclerosis Project and the WHO "Five Town Study", explores the structural changes in arteries that may determine the development of atherosclerosis, especially its early stages and its progression related to various contrasting socio-cultural settings and individual characteristics. In putting emphasis on the arterial changes that might indicate onset and progression of atherosclerosis and their correlation with environmental influences, the present protocol includes the use of light and possibly electron microscopy assisted by morphometry, histochemistry, immunohistochemistry and biochemical analyses, allowing an inquiry into structural and/or chemical changes, investigated only partially, if at all, in the two earlier international projects.

Besides obtaining information from contrasting populations, the reason for setting up a cooperative study is that any single investigator would have difficulty in obtaining a sufficient number of study subjects within a reasonable time because of the usually low mortality in young individuals and low autopsy rates, and also because accessibility to autopsy information and specimens is often limited. It is estimated that within three to four years, a sufficient number of observations could be collected in a multicentre cooperative study from countries with contrasting patterns of coronary morbidity, to obtain at least 20 cases in each age and sex subgroup.

2. OBJECTIVES

The general hypothesis underlying the study is that atherosclerosis begins in childhood and progresses through young adulthood into advanced lesions which may cause clinically manifest ischaemic disease in middle age and later. More specifically, it is assumed that certain features of the arteries or certain arterial lesions in young individuals predict the likelihood of the development of advanced lesions causing ischaemic disease later in life and that such features are different in populations with different frequencies of ischaemic diseases, particularly coronary heart disease. In summary, it is assumed that the arteries of young individuals show qualitative and/or quantitative differences which are related to the frequency of coronary heart disease in their respective populations.

Despite much earlier work, many questions remain to be answered, for example:

1. Can any changes be detected by morphometry, histochemistry, biochemistry, immuno-histochemistry and ultra-structural studies in the arterial walls of young individuals before the appearance of grossly visible atherosclerotic lesions? If so, do they vary in degree and kind between populations with high and low rates of coronary heart disease?

2. Does histo-morphometric and chemical evaluation of normal arteries and of macroscopic arterial lesions of similar type show differences between high and low incidence populations? If so, are these related to the mechanisms which lead to progression of atherosclerosis?

3. Within one culture, how are the arterial lesions related to age, sex, race and individual life habits, and how much do they differ between various cross-cultures?

The study will attempt to answer the following questions:

1. Do the arterial intimal lesions that are characteristic of later childhood (fatty streaks) progress into the lesions that, in adulthood, are closely associated with arterial occlusion and ischaemic disease (fibrous plaques)? At what age and under what conditions do "transitional" lesions (that is, lesions that have characteristics of both fatty streaks and fibrous plaques) occur? What are the topographical relationships of the different lesion types with increasing age?

2. If the progression hypothesized in (1) does take place, what are the earliest detectable changes in the fatty streak that indicate this progression? More specifically, are there differences in the lipid and/or lipoprotein composition and location, the cells and other formed blood constituents adhering to the endothelial covering, fibrin and platelet localization, monocyte/macrophage/foam cell population, smooth muscle cell number and structure, trace element and electrolyte composition, and connective tissue composition of the fatty streaks which are most likely to progress to fibrous plaques, as compared to fatty streaks which are not likely to progress to fibrous plaques?

3. Are there arterial lesions other than fatty streaks that progress to fibrous plaques? More specifically, what is the association of proliferative or insudative lesions with raised fatty or fibrous plaques.

4. What are the qualitative and quantitative relationships of the risk factors (identified by their association with clinically manifest adult coronary heart disease) to the localization, extent, and the gross, microscopic, and chemical features of atherosclerotic lesions in adolescents and young adults? Are there morphologic or biochemical lesion markers for hyperlipidemia, cigarette smoking, hypertension, male sex, or diabetes that can be correlated with the progression of lesions? If such markers are found, what mechanisms are suggested by which the risk factors accelerate atherogenesis?

5. Are there morphologic or biochemical markers which contribute to understanding the paradoxical excess of fatty streaks in young females over those in young males, even though adult males develop advanced lesions more rapidly and clinical disease earlier in life?

6. Can one identify, document, and quantitate changes in the lesion matrix which may have an important influence on progression?

3. ORGANIZATION

The need for interpreting morphological findings in relation to age, sex and contrasting types of human populations necessitates international collaboration, as well as cooperative efforts within a country, state or large city. Coordination between the collaborating centres and special laboratories is achieved through the coordinating centre and the management and data centre.

3.1 Collaborating Centres

At the collaborating centre one principal investigator must be responsible for all its work.

The centre must be affiliated with a large general and/or children's hospital and/or to a medico-legal autopsy service, especially to provide the cases for the younger age group and since deaths due to accidents and violence are an essential requirement of the study.

Each centre must have facilities for refrigeration of bodies as soon as possible after death. The centres collect the background information on the study subjects and their family, make the autopsies, and dissect, fix and/or freeze the arterial specimens, select specimens for the relevant histological sections and for blood and possibly other tissue analysis and ship arteries and specimens to the Processing and Receiving Centres. They should also send relevant data forms to the Management and Data Centre.

The collaborating centre must have facilities to fulfil the requirements for the whole study, for the proper preparation of the specimens, for bagging, freezing, and shipping the specimens. The collaborating centre must, during the whole study, preserve all material that has not been sent to a Processing and Receiving Centre.

3.2 Processing and Receiving Centres

These Centres are located in Malmö, Sweden, and Moscow, USSR, respectively. The Malmö Centre receives all fixed specimens, both gross and sections for histology, and the Moscow Centre all frozen specimens, both from arteries and other tissues. They are responsible for the processing and analysis of the specimens and for the storage of them during the whole study. They may collaborate with other laboratories for special preparations and analyses, e.g., enzyme- and immunohistochemistry, ultra-structural studies, image analysis and digitized morphometry. They are also responsible for checking the conformity of procedures and will work in close cooperation with the Coordinating Centre and the Management and Data Centre.

3.3 Coordinating Centre

This centre, which is based in Malmö, will supervise the quality of specimen processing and supply collaborating centres with technical advice and aid. It will keep records of accession numbers and will work in close contact with all the collaborating centres, Processing and Recenting Centres, and the Management and Data Centre to help insure the functional and quality performance of the program.

3.4 Management and Data Centre

This centre will be based at WHO/HQ, Geneva and will assist in the design of the study, develop the internal checks and quality control measures, design the data recording and the data processing and preservation methodology for the major determinations to be done in all cases. It will analyze the data and program reports and manuscripts concerning the major aims.

3.5 Meetings of principal investigators

These will review and decide the planning and execution of the project, monitor and evaluate progress, exchange experience and information, review the recommendations of the Steering Committee, and agree timetables.

3.6 Steering Committee

The Steering Committee will carry forward the work of the project on behalf of the principal investigators.

4. METHODS

4.1 Choice of study sites

Cross-cultural comparisons require study sites contrasting in living habits. Since there are marked differences in coronary heart disease frequency, not only between but also within the developing and already industrialized parts of the world, the project should be undertaken in several sites representing characteristic living patterns in a variety of cultural and ethnic settings. Even within the same country, social contrasts may provide such "natural experiments". However, living conditions are undergoing rapid changes in many regions, a fact that has to be taken into account when defining the study sites.

Within these broad cross-cultural requirements, the choice of a given study site is further determined by the availability of a team of investigators both interested and experienced in this research field, as well as adequately staffed and equipped to carry out the required work for the necessary period of time, and able to secure local financial support for the study. Places in which background information on the population is available or can be collected, and in which personal history data of the studied subjects are readily available or can be collected, have priority. The extent to which these conditions are met must be assessed in each situation between representatives of the prospective collaborating centre and the coordinating centre.

4.2 Definition of study subjects

Included in the main study are subjects of both sexes dying between the ages of 5 and 34, with emphasis on untreated subjects and victims of fatal accidents. However, subjects dying of any other well-defined condition should also be included. Preference should be given to sudden unexpected deaths without previous medical treatment and such cases should be possible to identify from all collected series. Time between death and autopsy start must be less than or equal to 72 hours (exclude obvious putrefaction).

Efforts should be made to obtain information on the personal and family history of all deceased subjects.

4.3 Epidemiological information

Environmental and morbidity differences between populations are the platform for comparing the morphologic findings. It is therefore as essential to secure a maximum of epidemiological information on the background populations from which autopsy cases are derived, as it is standardized and comparable anatomical and laboratory methods. It is not possible to include in the present study a cooperative epidemiological study of coronary heart disease and related socio-environmental factors; however, all existing information on these issues should be collected in each collaborating centre. The principal investigators in all centres are therefore advised to assure the participation or, better still, the partnership of an epidemiologist (cardiovascular) from the area (or thoroughly acquainted with the study area), for producing a comprehensive review of the epidemiological characteristics of the background population. This information should be derived from mortality statistics, health surveys carried out earlier, and the known demographic data for the area or country. A list of the desirable information on the background populations is given below:

(i) Demography
- Size
- Age and sex composition

(ii) Mortality
- Age and sex specific mortality
- All causes
- All CVD
- Ischaemic heart disease
- Stroke
- Hypertension

(iii) Morbidity
- Incidence of myocardial infarction

(iv) Risk factor levels
- Blood pressure
- Smoking rates
- Serum lipid levels

(v) Anthropometric data (children and adolescents, adults)

- Body weight
- Height
- Body mass index

(vi) Food and other consumption

- Energy intake per capita
- Total intake
- Protein percentage
- Fats percentage
- Carbohydrates percentage
- Types of fat used
- Milk consumption per capita
- Alcohol consumption per capita
- Tobacco consumption per capita

However, since the comparability of retrospectively gathered information is difficult to assess, a detailed description of the sources of all information and a careful narrative of the epidemiological profile of the background population is of greatest importance. This will then be used in the final analysis of the study for classifying the background populations into qualitative categories, based on demographic, mortality, morbidity, anthropometric, risk factor, and consumption criteria.

4.4 Individual information: personal history of study subjects

Subject's family name (surname); given name; sex; date of birth; place of birth; country of birth; duration of present residence (if immigrant, the duration of stay in the country); ethnic group (if pertinent); occupation. If possible, and especially in children below 15 years of age, information on parents, as above, as well as their medical history.

Has subject ever had any serious disease? Has subject been treated in hospital? Has blood pressure ever been measured? Result? Has high blood pressure ever been found? Smoking habits? Alcohol habits?

In females past menarche, number of pregnancies and abortions; use of oral contraceptives.

4.5 Collection and preparation of arterial and other specimens

The main specimens in the study are the aorta and the coronary arteries but also postmortem blood and kidney tissue is collected from all cases. Optional procedures included are collection of adipose, liver and myocardial tissues.

The thoracic and abdominal aorta is removed in one segment, cleaned from adventitial fat, opened and divided longitudinally. The left half is fixed, put in a plastic bag and shipped to a central laboratory, the Receiving and Processing Centre (RPC) in Malmö, for macroscopical staining and evaluation. From the right half, three standard specimens as well as one lipid streak and one fibrous plaque are taken for histological evaluation and two specimens for chemical analysis. The main part of this aortic half is left for the collaborating centre for its own studies, preferably organized in collaboration with other centres.

Of the coronary arteries, the right one is prepared as the aorta, formalin-fixed and shipped in a plastic bag to the Malmö RPC for macroscopical staining and evaluation. The left circumflex artery is also removed opened in one piece, cleaned from adventitial fat but then deep-frozen and shipped to the Receiving and Processing Centre (RPC) in Moscow, for chemical analysis. The left main and descending coronary artery is removed from the heart unopened, fixed in formalin and then cut transversely in 5 mm intervals. Section number 3, which is taken just below the orifice of the left circumflex branch, is sent to the Malmö RPC for further processing, but sections numbers 1, 5 and 7 are processed at the collaborating centre.

Postmortem blood is used for risk factor analysis, i.e., determination of total lipid, HDL and LDL cholesterol, triglycerides, thiocyanate levels as well as measure glycosylated hemoglobin in postmortem cells. Frozen serum and blood cells are analysed at the Moscow RPC, which is standardized against one of the WHO reference laboratories for lipid analysis. However, each collaborating centre is encouraged to perform its own chemical analysis and standardize it against the Moscow RPC.

One slice of kidney tissue including both cortex and medulla is taken from each kidney and formalin-fixed. The specimens are then shipped to the Malmö RPC for processing.

Of the optional tissues, liver and adipose tissue is analysed for various types of lipids and fatty acids whereas myocardial tissue is analysed for signs of hypertrophy, ischaemia or any other damage.

A summary of the type of evaluation for different types of specimens is given in Table 1.

TABLE 1

Type of Evaluation for different types of specimens

Type of specimen	Macroscopic	Microscopic	Biochemical	Enzyme-histochemical	Immunohistochemical	EM
Aorta thor.	+	+	+	Opt.	Opt.	Opt.
Aorta abd.	+	+	+	Opt.	Opt.	Opt.
Coron. LAD	-	+	-	Opt.	Opt.	Opt.
Coron. LC	-	Opt.	+	-	-	-
Coron. RC	+	+	-	-	-	-
Other artery	Opt.	Opt.	Opt.	Opt.	Opt.	Opt.
Blood	Not applic.	Not applic.	+	Not applic.	Not applic.	Not applic
Kidney	Not applic.	+	-	-	-	-
Adipose tissue	Not applic.	-	+	-	-	Not applic
Liver	Not applic.	-	Opt.	Opt.	-	-
Myocardium	Opt.	Opt.	Not applic.	Opt.	Opt.	Opt.

Opt. = optional
thor = thoracic
LC = left circumflex
Not applic. = not applicable
abd = abdominalis
RC = right coronary
EM = electron microscopy
LAD = left anterior descenders

4.6 Analysis of specimens

Gross arteries, i.e., the left half of the thoracic and abdominal aorta, and the right coronary artery are graded for extent and type of lesion after staining with Sudan IV. The staining technique is as described by Uemura et al. (Bulletin of the World Health Organization, 31:297, 1964).

The grading is done by a team of trained graders with no knowledge of the origin of the specimens and the results recorded on special forms. The stained specimens are also photographed and the pictures are analysed using digitized automatic scanning techniques. The arteries are also investigated by soft X-ray for presence of calcifications. The intact specimens may also be analysed by other type of naked eye or automatic procedures providing they are kept intact during the whole study.

Evaluation of the histological sections from aorta and the left anterior descending coronary artery is likewise done by a team of specially trained pathologists but also by computer assisted automatic image analysis at special centres. The sections are stained both by haematoxylin-eosin and several special stains as well as with lipid stains. If possible, histochemical and immunohistochemical methods are also applied. The definite methods for analysis of the histological specimens are to be worked out during the pilot study.

The chemical analysis of arterial tissue and postmortem blood is performed at a central laboratory, the Moscow RPC. The collaborating centres are encouraged, however, to perform such analysis themselves on aortic tissue and blood. Adipose and liver tissue are chemically analysed for lipids, but only as an optional procedure.

Fixed kidney tissue is processed in order to study the structure of its arteries which may reflect the blood pressure level of the individual. In the evaluation the method described by Tracey is to be used (American Journal of Epidemiology, 91:25, 1970). In principle, it measures the ratio of intimal thickness to outer diameter on PAS-Alcian blue-stained paraffin sections. 40-80 arteries per case are measured. Abnormalities of arteries with outer diameters of 140-300 um correlate well with age while those of 90-120 um can be used to estimate prior levels of blood pressure.

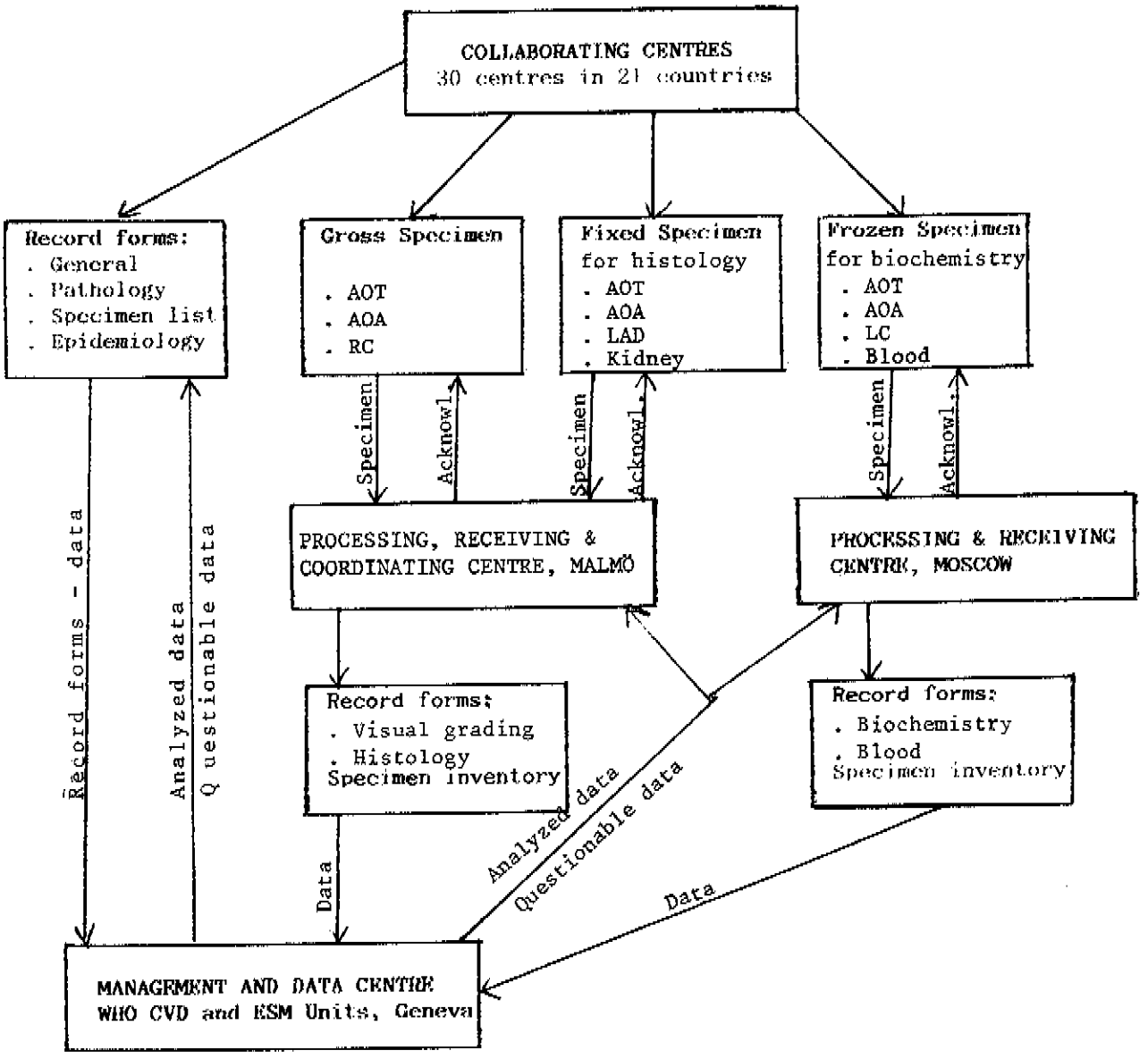
5. INFORMATION FLOW AND DATA PROCESSING

The summary of information flow is given in Figure 1 for the core study, and in Figure 2 for the optional studies. It shows the tasks of the different centres and the information flow from and to the collaborating centres and the cooperation within centres.

FIGURE 1

PATHOBIOLOGICAL DETERMINANTS OF ATHEROSCLEROSIS IN YOUTH (PBDAY STUDY)

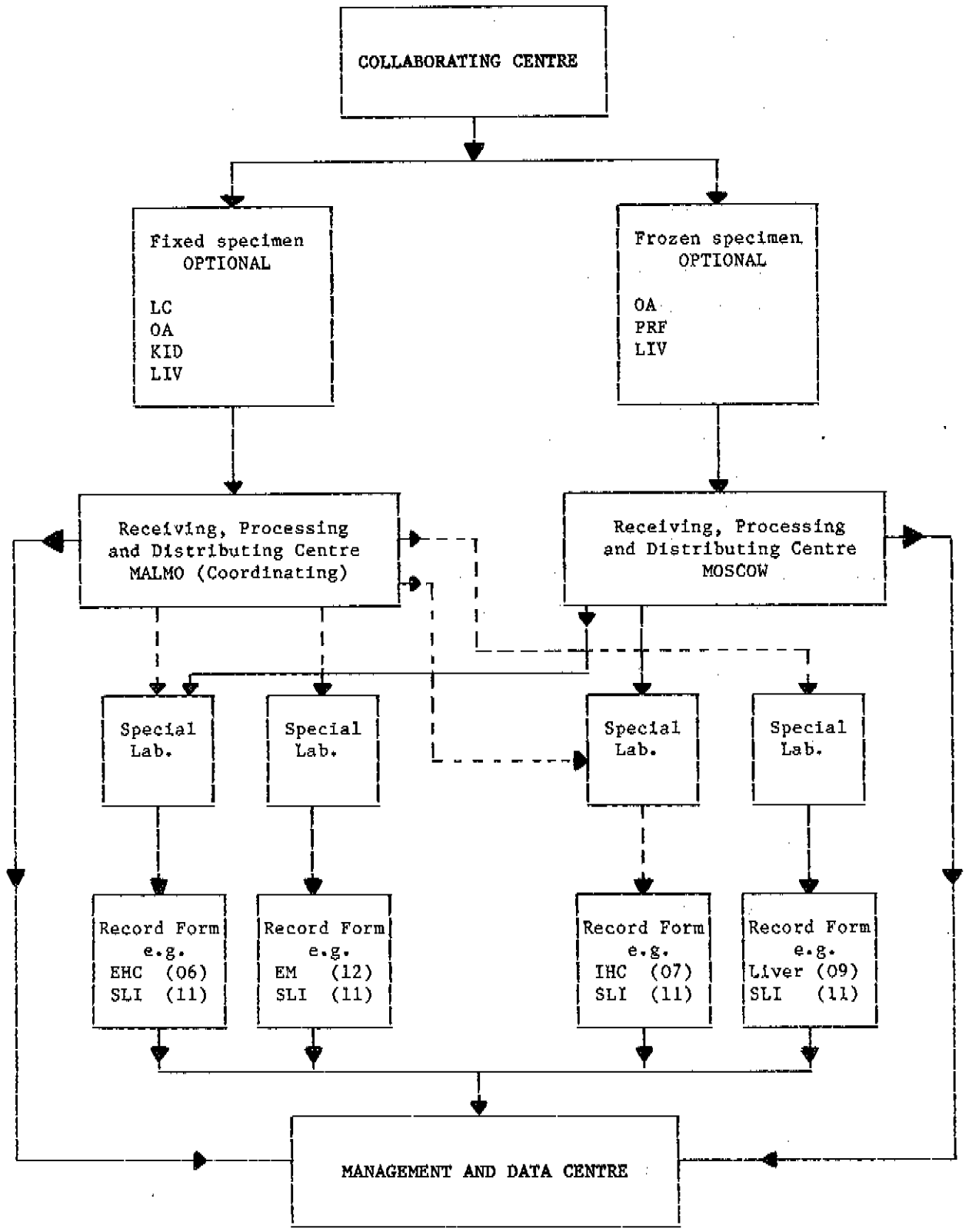
Summary of information flow in the core study



Abbreviations: AOT = aorta thoracica
AOA = aorta abdominalis
RC = right coronary artery
LAD = left anterior descendens coron.
LC = left circumflex coron.

FIGURE 2

SUMMARY OF INFORMATION FLOW IN THE OPTIONAL STUDIES



The data management system consists of logically sequenced operational components viz. data collection, entry, editing, monitoring, storage, and retrieval. This will allow for the effective and efficient handling of study data for the assurance of high quality collection of the original observations by the Collaborating Centres, the provision of aggregated data for specified analyses and for the accomplishment of archival storage of the data. The components function simultaneously and there will be continuous interaction between the data collection points and the central management facility.

6. COLLABORATION WITH THE US PDAY STUDY

The Steering Committee of the PBDAY and PDAY studies, respectively, should consider some form of associate membership similar to the practice in the MONICA study. It means that the Steering Committee of each study is entitled to receive all material from the other study, to participate in meetings at its own expense, etc. Although PDAY study is more detailed than the PBDAY study, close liaison between the two should be of great scientific value.

In order to ensure as good comparison as possible between the PBDAY and the PDAY studies, the collection and preparation of the aorta and the right coronary artery, used for macroscopical grading, are identical. The evaluation is also the same and specimens may be interchanged between the two studies in order to allow absolute comparison of results. Likewise, the specimens for histological examination from the aorta and the left anterior coronary artery are taken in identical places and processed in the same way. The same holds for specimens for chemical analysis, i.e., pieces from the aorta and the left circumflex coronary artery. These so-called core specimens are all processed in central laboratories which will standardize their analytical methods against those used in the other study. Likewise, chemical analysis of blood samples will be compared. Image analysis of pictures of stained arteries are supposed to be performed at one central place for both studies.

7. PUBLICATION POLICY

7.1 Reports based on the common protocol

7.1.1 Preparation

The Steering Committee, acting on the advice of the principal investigators, will appoint writing groups to prepare reports for publication on specific topics as results become available. All publications of this type will be reviewed and approved in writing by all principal investigators before they are submitted for publication.

7.1.2 Authorship

Reports based on data generated by the Receiving and Processing Centres on specimens collected by all collaborating centres will be submitted for publication under the name of the WHO/ISFC Study on Pathobiological Determinants of Atherosclerosis in Youth. Each such report will include a full note or an appendix listing the principal investigators and their institutional affiliations, possible grant numbers, and other professional staff members of the centres designated by the principal investigators. The appendix or footnote will also list the persons preparing the report.

7.1.3 Reprints

A limited number of reprints will be purchased for distribution to collaborating centres but the address for reprint requests from readers will be that of the Steering Committee.

7.2 Reports based on special studies by the centres

7.2.1 Preparation

Principal investigators and their associates in both the collaborating and other centres are expected to conduct studies, not part of the common protocol, which use material collected by all collaborating centres. These studies may include tissue analysis, data analysis, methodological development, or other work which is meritorious and which should be published.

Before each report of this type is submitted to a journal for publication, it will be reviewed by the Steering Committee for scientific quality, relevance to the overall project and appropriate acknowledgement of the project participants. If the Steering Committee does not approve of its content, the principal investigators may still submit the report for publication, but will not acknowledge support of the overall project.

7.2.2 Authorship

The reports of such research will be prepared by the principal investigator or by his/her designated alternate. Each such report will acknowledge the source of the material as the WHO/ISFC Study on Pathobiological Determinants of Atherosclerosis in Youth. It will include a footnote or an appendix listing the participants, their institutional affiliations, and possible grant numbers.

7.2.3 Reprints

The participants who publish reports of this type will provide a limited number of reprints to the Steering Committee but will use their own addresses for reprint requests.

7.3 Reports of research conducted by a principal investigator on his own material

7.3.1 Preparation

Any principal investigator, or his designated alternate, may at any time prepare reports of research conducted on material collected by his own laboratory. The principal investigator will inform the Steering Commttee of such reports, but the Committee is not required to review them before submission.

7.3.2 Authorship

The participating investigator who conducts the research and writes the report will designate the co-authors other than himself. He will cite participation in the overall project but does not need to list all participators.

7.3.3 Reprints

Each participating investigator who writes a report of this type is requested to provide a limited number of reprints to the Steering Committee.

8. TIMETABLE

The draft Protocol and Manual of Operations of the study will be sent out in April 1986 to the collaborating centres. Within a month, approximately 10 kits including record forms will be received by collaborating centres for the pilot study. It is hoped that the pilot study will be concluded within three to four months. Five cases from each centre will give the Management and Data Centre the possibility to evaluate the study methods and processes. Based on these results and on the comments of the collaborating centres on the protocol and manual the Steering Committee will decide on finalization of the protocol and manual in November 1986. The main study will commence in January 1987. The revised record forms, protocol and manual of operations will be distributed preceding the commencement of the study, together with the necessary kits.

9. FUNDING

The main part of the costs for each collaborating centre is expected to be covered from local sources. The World Health Organization is prepared to assist the collaborating centres in grant applications.

WHO may, subject to the availability of funds, provide a small grant for some collaborating centres, based on individual contracts between WHO and the centre. This will enable the centre to order some equipment and material through WHO or use the funds for local purchases. The collaborating centre must make sure that their basic needs for the study are covered.

The World Health Organization may also provide funds, subject to availability, for the Receiving and Processing Centres for equipment sent to the collaborating centres (kits, plastic bags, sealers, styroform containers, etc.), for chemicals, glassware etc., used in the processing of specimens, and for part of the staff needed. It will also provide support for meetings of the Steering Committee, for possible site visits, and for the occasional meeting of the principal investigators.

The World Health Organization will also be responsible for funding the Management and Data Centre at WHO headquarters.

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