Original Article

Laboratory Protocol and Development of Biorepository for Epidemiological, Rural DERVAN Cohort Study in KONKAN Region of India (DERVAN-2)

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Abstract

Background: According to Developmental Origins of Health and Disease (DOHaD) hypothesis the seeds of adult non communicable diseases like diabetes, hypertension and cancer are sown in foetal life. DERVAN cohort is a longitudinal study and was launched in the Kokan region of Western Indian state of Maharashtra. It recruited 1520 adolescents girls aged (16-18y) as well as their parents. At base line it measured various analytes in the blood, saliva and urine which together with additional data such as body composition, cognition, and socioeconomics will provide the baseline health status of adolescent girls. We plan to follow the subjects systematically for next 20 years where more biological samples are expected to be collected. Hence the development of bio-repository formed a critical component of our study. We measured biochemical, nutritional (micronutrients), hormones, trace elements in adolescent girls from longitudinal DERVAN cohort and setup bio-repository.

Methods: At base line 36 ml of blood, urine and saliva in fasting state was collected from girls. We also collected blood (20 ml), urine, saliva in random state from both the parents. In addition some analytes were measured immediately and remaining samples (30 aliquots for girls, 12 aliquotes for each parent) were stored in -80ºC freezers. All the samples were collected at the research centre to ensure quality and longevity. External as well as internal quality control protocols were followed. The Bio-repository is now in place for long term storage.

Conclusion: The protocol adopted by us has been found to be working smoothly and can guide many researchers working in resource limited settings.

Key words: Bio-repository, Rural, Cohort study, India, KONKAN

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Introduction

India is witnessing a rising prevalence of Non communicable diseases (NCD). This is attributed to change in lifestyle and physical activity. This is clearly visible in urban settings which have undergone rapid nutritional transition over last 2 decades. But the NCDs are also on the rise in rural India[1] and they are becoming the main cause of morbidity and mortality. According to Developmental Origins of Health and Disease (DOHaD) theory, roots of NCDs are intrauterine as well as intergenerational[2]. Many cohort studies, both retrospective[3-4] and prospective[5-8] have been set up in India to test the DOHaD hypothesis. India is demographically very heterogeneous. The research findings of these cohort studies reflect the continuous challenges of cultural and socioeconomic diversity that influences food choice, nutritional intake, and health status among population groups in different regions of India. All of these studies emphasize the important role of maternal nutrition both before conception as well as during pregnancy.

BKL Walawalkar hospital was established in the year 1996 in a village of Dervan located in the KONKAN region of the Maharashtra state of India. The hospital runs various holistic programs for children, adolescent girls, newly married girls and pregnant women. Since inception, women's health has been a prime area of interest. Comprehensive health education is provided and various investigations are carried out. These programs encompass children, adolescent girls, newly married, and pregnant women. Counselling, holistic education, and medical treatment if needed is provided free of cost. Various hospital and community based cross sectional as well as observational studies have demonstrated the presence of malnutrition across the entire life cycle [9-11]. In addition we have also observed a rising prevalence of NCDs (lean diabetes and hypertension)[12]. This suggests an intergenerational link between malnourished mother and her offspring. NCDs are known to have prolonged subclinical phase of progress lasting for years, hence a prospective cohort study investigating these conditions was set up to get insights to NCDs and in particular diabetes in rural Konkan. This thought laid to setting up of DERVAN cohort of adolescent girls. It would test the hypothesis that poor physical growth and poor nutrition in adolescent girls increases the risk of NCDs, in particular the risk of diabetes in their adulthood and in their off springs[13]. Baseline recruitment of 1520 adolescent girls is complete and subsequent annual follow ups are in complete progress. As of December 2023, 624 mothers, and 201 fathers have been recruited. Biological samples collected and analysed in this cohort study have provided a wealth of information about the current health status of adolescent girls in the Konkan region. This along with contextual features such as body composition, cognition, socioeconomic conditions, air and water quality, personal hygiene practices, food access, and educationare helping us in determining the future risk of diseases.

This manuscript describes the laboratory protocol for the current stage (stage-1) and also proposes protocols for the subsequent stages.

Materials and Methods

Field Activities

Our team of social worker and medical officers visit the villages in the study area. Meetings are conducted at schools and local government offices, women's self-help groups, and also at Anganwad is (rural childcare centre). Information about the project is provided to attendees by slide shows as well as printed pamphlets. Any doubts raised are clarified immediately by the field team members. This exercise is continuous since the time of recruitment.

Ethics

The study was approved on 21st of July 2018 (Ref BKLW/RMC/IEC/16/2018(07) by the ethics committee of BKL Walawalkar Rural Medical College and Hospital.

Consent of participants

Appropriate assents as well as consents were obtained from adolescent girls. For girls below 18 years of age additional consent was obtained from parents.

Biological sample collection, processing and storage protocol: adolescent girls (AG)(Figure-1)

Blood pressure was measured prior to blood collection. We did not collect blood in case the subject had hypotension. Fasting blood was collected in supine position to avoid fainting. Blood collection was performed by venipuncture using a vaccum collection system, after asepsis of blood collection site with alcohol. About 36 ml blood was collected in appropriate vacutainers (EDTA, Plain and Trace element) by phlebotomist. Complete blood count (CBC) and HbA1c are measured from whole blood. Another 2 ml of whole blood was aliquoted into 2 ml cryovials and stored into -80°C freezer. Remaining blood was centrifuged at 3000 rpm for 15 minutes at 4°C in a cooling centrifuge. Aliquots of plasma, serum and packed cells were prepared and stored in

appropriately labelled cryovials in the bio-repository. Two hours postlunch, we collected blood sample for the measurement of post prandial glucose level.

Urine

Twenty ml midstream urine was collected in a 40 ml sterile container and then transferred into glass tubes and centrifuged at 1500 rpm for 10 minutes at 4ºC. Urine routine examination was done on the day of sample collection. Additionally, two 2 ml aliquots of urine were prepared and stored in the bio-repository.

Saliva

Unstimulated saliva samples were collected in the morning. Research participants were asked to rinse their mouth with water 5 minutes prior to the collection. Participants were asked to avoid use of mouthwashes, lipsticks and consumption of food, drinks and chewing gums before submitting saliva sample. Ten ml saliva was collected in a 25 ml wide-mouthed sterile polypropylene container. Saliva was centrifuged at 1000 rpm for 5 minutes at 4ºC and aliquots were prepared. Bar code and UID was applied to the aliquot tubes. Immediately after blood collection, breakfast was served to girls and then they proceeded for other investigations. All the samples collected were stored at -80°C for further investigations.

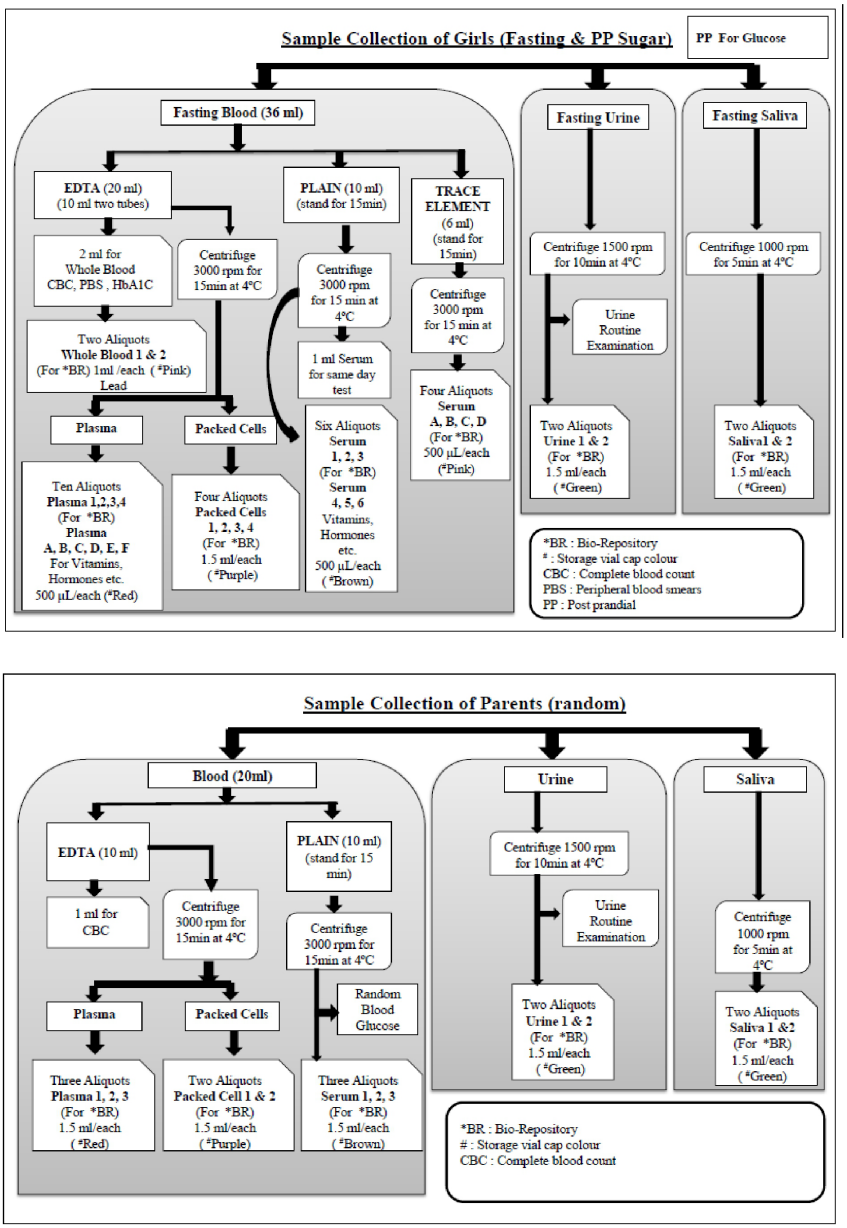

Figure 1: Sample collection

Biological sample collection (Parents) (Figure-1)

Random biospecimens were collected.

Blood

Blood sample was collected from parents in supine position. About 20 ml of blood was collected in appropriate vaccutainers (EDTA and plain). CBC was performed on the same day. Remaining blood was centrifuged at 3000 rpm for 15 minutes.

Urine

Collection, processing and storage of urine samples were performed according to the standard protocol which was same as that for adolescent girls.

Saliva

Parents were asked to rinse their mouth 5 minutes prior to the sample collection. In addition, they were advised to not chew tobacco or chewing gum before providing sample. Unstimulated random saliva sample (approximately 10 ml) was collected in a sterile 25 ml polypropylene container. Processing, labelling and storage of the sample was performed according to the standard protocol.

All the samples collected were stored at -80°C for further investigations.

Sample storage

A separate 150 square feet air conditioned room with generator back up has been specially allocated for the bio-repository. We have four -80°C freezers (Thermo fisher scientific) each with 4 chambers. Digital temperature record is in-built and is displayed continuously and an alarm system monitors the fluctuations. It also generates temperature logs which are minute interval apart and can be backed up externally. Figure 2 shows Summary of daily mean temperature of the freezer for month of January 2020 while Figure 3 shows summary of monthly mean temperature with standard deviation over 17 months. Freezers can be accessed only with user ID and password. Event logs (opening and closure, battery, power backup) are generated. Freezers are linked to hospital power alert system which sends alerts in case of freezer failure or power failure.

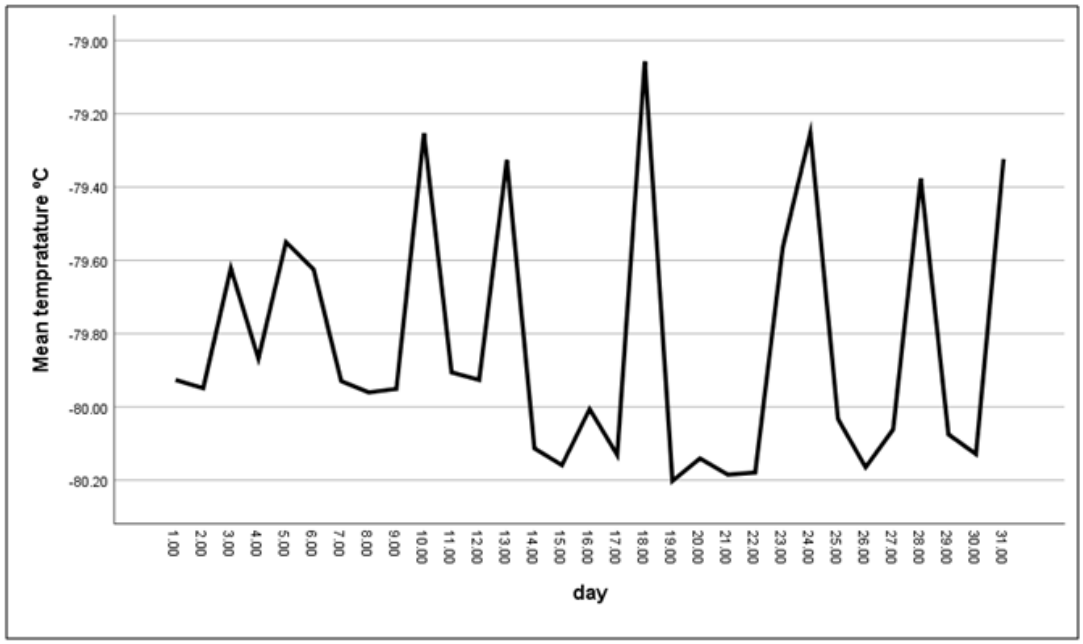

Figure 2: Daily mean temperature of the freezer (January 2020)

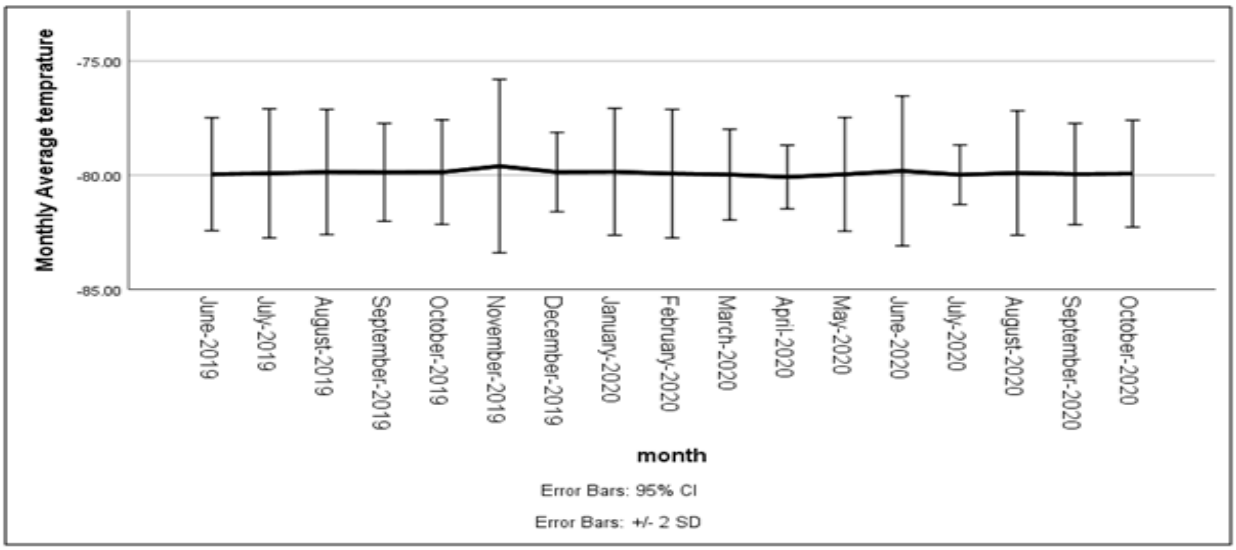

Figure 3: Monthly mean temperature variation with standard deviation (June-2019 to October-2020)

There are total 30 aliquots for each adolescent girl. Sample cryovials are stored in cryo boxes designed to hold 81 vials each. Each box is specified to hold only one type of aliquot and it is labelled accordingly. Colour of the aliquot vial caps identifies the aliquot type as whole blood, plasma, serum, packed cell, urine and saliva (Figure-4). Similar storage protocol is used for parents biospecimen, where number of aliquots is 12. Cryo boxes are placed in specially designed racks with capacity to hold 5 boxes. Parents samples are stored chronologically and according to UID in order to optimise the storage space.

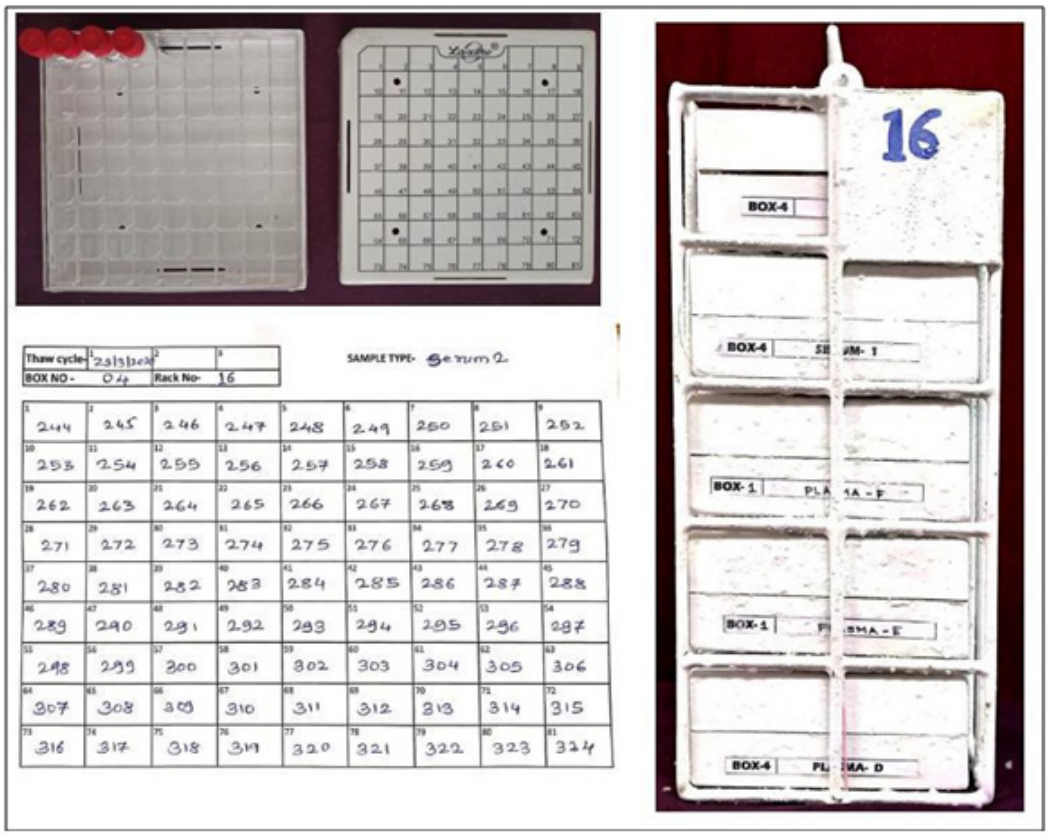

Figure 4: Sample Storage

Sample storage and retrieval information system

In order to trace any sample, we need 6 attributes: UID, freezer number, chamber number, rack number, box position and sample position in the box. We are storing this information in an excel spread sheet as well as in the form of paper maps which are matched before retrieval.

Biospeciman analysis (Table-1)

Certain measurements were performed on the same day of blood collection (e.g lipid profile, blood glucose, LFT, RFT, TFT calcium, uric acid, albumin, total protein, vitamin D and vitamin B12). Others are done as per the availability of reagents and assay kits. Kits are stored as per protocols specified by the manufacturer. Measurements of analytes were planned such that maximum number of tests was done per aliquot. This helps in reducing the number of thaw and re-thaw cycles. These cycles are documented in the sample storage paper maps and corresponding Microsoft Excel spread sheet.

Table-1: Details of analytes measured and instruments used

Analytes	Measuring Instrument
Glucose (fasting and post prandial), Liver Function Test (Bilirubin, total protein, albumin, globulin, SGOT,SGPT), Renal Function Test (RFT), Lipid profile, Uric acid, Calcium, hs CRP, Magnesium, Phosphorus Urine routine Examination	Erba 200 Clinical Chemistry Auto-Analyser
Vitamin B12, Vitamin D, Thyroid Function Test (TFT), Folate, Ferritin, TIBC, Insulin, Parathyroid hormone (PTH), Homocysteine, Follicle-stimulating hormone (FSH), Luteinizing hormone (LH)	Abbott Architect Immunoassay
HbA1c	Bio-Rad D10 HPLC system
Haemogram	Horiba Yumizen H 500
Vitamin B1,Vitamin B2, Vitamin B6, B12, Folate, Vitamin C, Vitamin E, Growth Hormone (GH), Insulin like growth Hormone (IGF-1), Leptin, Calcitonin, Retinol binding protein (RBP)	ELISA (Bio-Rad micro plate reader and washer)
Zinc, Copper, Manganese, Selenium, Lead, Chromium, in Water and serum	Shimadzu Graphite Furnace AAS 6080

Quality control practice

Every measurement done on the sample undergoes internal as well as external quality control (QC). We are using instrument specific internal controls. We are part of External Quality Assessment Scheme (EQAS)[14]. Quality control data for the analytes measured so far has been described in Table-2. External standard controls by Seronorm (reference number -201405) were used for copper, zinc and manganese.

Table-2: Quality control (lab investigations)

Parameters	Intra-assay CV (%)	Inter-assay CV (%)
Haemoglobin (gm%)	2.90	4.1
Ferritin (ng/ml)	8.03	2.92
Fasting Glucose (mg/dl)	3.83	4.37
HbA1C (%)	4.3	2.86
Urea (mg/dl)	4.03	5.94
Serum Creatinine (mg/dl)	6.0	4.40
Uric Acid (mg/dl)	9.3	6.77
Bilirubin-Total (mg/dl)	2.03	6.93
Total Protein (g/dl)	2.85	6.28
Albumin (g/dl)	1.8	3.39
SGOT (U/L)	9.14	5.33
Alkaline Phosphatase (U/L)	3.19	7.39
Cholesterol (mg/dl)	3.5	5.27
Triglycerides (mg/dl)	6.86	5.73
HDL (mg/dl)	7.04	3.80
Insulin (mIU/L)	6.01	2.03
Homocysteine (µmol/L)	7.9	3.55
Thyroid Stimulating Hormone (µIU/mL)	2.39	4.22
Pyarathyroid Hormone (pg/ml)	7.81	2.14
Testosterone (nmol/l)	11.05	1.25
Vitamin D (ng/ml)	4.62	6.55
Vitamin B_{12} (pg/ml)	5.3	2.53
Folate (ng/ml)	8.5	3.29
Calcium (mg/dl)	2.85	6.18
Leptin (ng/ml)	9.24	11.15
Insulin like Growth Factor-1 (ng/mL)	11.6	8.29
Growth Hormone (ng/mL)	10.36	7.31
Triiodothyronine (T_3) (ng/dL)	4.72	6.02
Thyroxin (T_4) (µg/dL)	4.68	2.41
Magnesium (ppm)	15.8	16.44
Phosphorus (mg/dl)	5.39	8.25
Zinc (ppm)	25.77	10.0
Copper (ppm)	10.80	7.6
Manganese (ppb)	8.10	1.31

Documentation and laboratory procedure

Records of all laboratory procedures and standard operating procedures (SOP) are maintained in the laboratory manual and they are subject to periodic review. Log books and records are maintained for equipment maintenance, supplies of reagents, test procedures and staff responsibilities.

Data management

Our cohort study data is stored in MS-Access data base. Data management protocol has been submitted as an additional manuscript together with this manuscript. A user friendly data management system is already in place. The laboratory component of our study is also a part of this system.

Discussion

We have described the laboratory protocol for the cohort study in one of the most resource limited and understudied region of India. From the inception of the hospital in 1996 our teams of social workers and medical officers has built a strong network with the villagers in the vicinity. They visit day care centres, primary health centres (PHC), women's self-help groups. Holistic education camps are also conducted. The Institute previously had organized adolescent girls anaemia detection camps in schools and colleges. Thousands of girls were screened for anaemia and related disorders. A pilot study measuring micronutrients in blood was also conducted before establishing a cohort.[11] Thus our community is not averse to giving blood sample, hence there was no hindrance for the assent and consent from the girl as well as her parents for participation in our study which is carried out among healthy adolescent girls who are seemingly normal. With the advent of new technologies home based collection approach has been suggested in developed world[15-16] but in large scale epidemiological study such as ours, logistics of blood collection and transportation of biospecimens requires a lot of consideration since most of the biospecimens have to be transported within a defined temperature range to the testing laboratory to preserve their integrity. Thus we decided to collect samples at the institute to avoid confounding issues of transportation and cold chain issues related to collection and processing of samples. Though the protocols and the procedures described above may look very straight forward, we would like to reiterate the fact that as our hospital is located in the midst of all villages, it was possible for us to get the girls to the institute for sample collection. Our systematic process thus ensured quality. Separate space measuring 1500 square feet as well as dedicated staff has been allocated for this research project. This is helpful in preventing interference with routine hospital work. Our laboratory instruments set up is completely separate from hospital. All the instruments are under maintenance contracts. Our laboratory team is well trained to perform the laboratory procedures.

Sample identification is the most crucial part of bio-repository so that they are accurately linked to an adolescent girl and her parents. Bar-coded labels are used which are resistant to fluctuations in temperature and humidity. Many a times long term storage of samples leads to label damage. We are putting an extratrans parent adhesive tape on the sample vial as an additional precaution. Our subject identities are kept confidential. Samples are identified only by the UID and subject name initials and family member code (AG-for adolescent girl, FATH- for father and MOTH-for mother)(Figure 5). Initially we had thought of identifying father by code 'F' and mother by code 'M' to optimise printing cost but this was prone to create a confusion at the time of sample retrieval by equating these codes for gender of the subject (F for female and M for male). Such minute attention to detail is extremely crucial for the long term cohort study such as ours, where staff turnover are always likely. Hence we simplified the coding system. Some bio-repository management software programs are available freely in public domain but they could not be customised for our protocol. We designed our own system using MS-Excel to manage the samples.

Figure 5: Barcode labelling

Our progress of the project and interim results have been presented to an external review committee which meets twice a year. Additionally, scientific

advisory committee of the institute also conducts annual review. Since this is a hypothesis driven cohort study we are unable to report detailed results at this time. But we have shown relevant results of quality control and freezer temperature variation which are crucial to our study.

One year follow up (Figure-6)

We are planning to collect 16 ml (EDTA and Plain) of blood, urine and saliva at 1 year follow up. We will measure glucose, Hba1c, insulin, and CBC. Remaining blood will be aliquoted for the bio-repository.

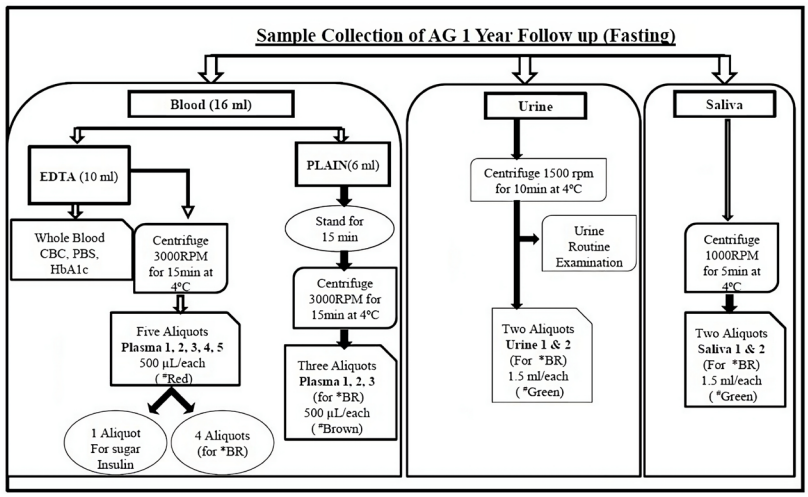

Figure 6: Sample collection at 1 year

Future plans (Figure-6)

Reconsenting process will be initiated once the girls reach adult age of 18 years. In the event of marriage, role of the husband and the in-laws becomes very important for the continuing participation of our enrolled adolescent girl subjects in the study. Though their consent is not mandatory, we will seek their cooperation by personal counselling sessions. It is common for Indian women to leave their place of origin after marriage. However, we do not expect this migration to affect our study as Indian women often return to their maternal home during pregnancy and subsequent delivery.

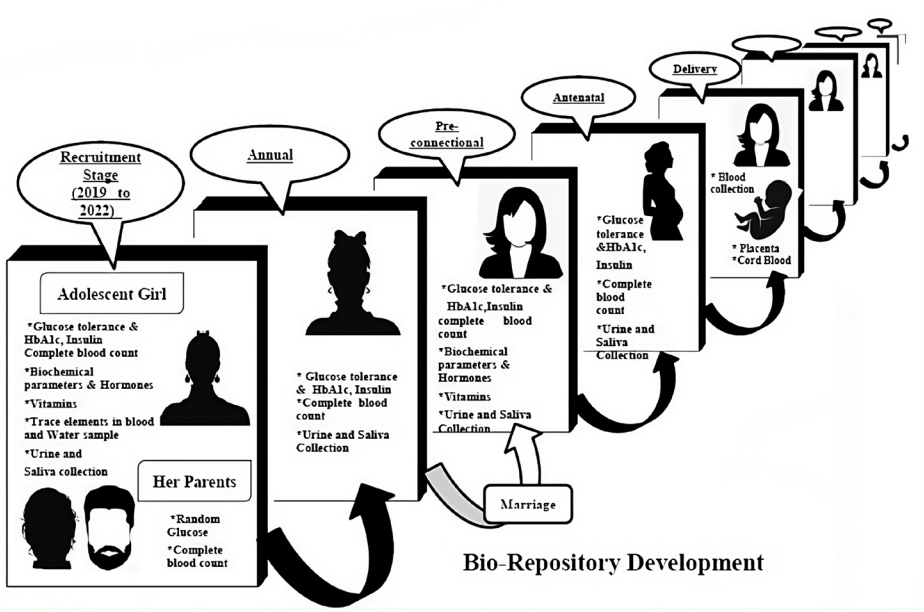

Figure 7: Future plans upto delivery

We are planning to follow these girls and their offspring's longitudinally over the next 30 years. We intend to collect biological samples at various stages: preconception, pregnancy, maternal and cord blood at delivery. At each stage we intend to collect blood for CBC, fasting glucose, HbA1c, insulin and few more tests which will evolve from the analysis of baseline data. We are storing packed cells and saliva especially for genomic and epigenetic studies which could be of tremendous value in the future.

Though our institute is well equipped with state of the art facilities, it is located in a remote rural region. Many a times there are inordinate time delays in the delivery of laboratory supplies (labels, reagents, vials, and instrument accessories). On the other hand our holistic approach towards the community helps us in the recruitment of the cohort.

In summary, our protocol for laboratory management and bio-repository development can be used as a guiding template which can be adapted to any resource limited setting.

Conflict of interest: None

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List of Abbreviations: NCD: Non communicable diseases, DOHaD: Developmental Origins of Health and Disease, CBC: Complete Blood Count, SGOT: Serum Glutamic Oxalo Acetic Trans Amainase, SGPT: Serum Glutamic Pyruvic Trans Amainase, UID: Unique Identity Number, EDTA: Ethylene Diamine Tetraacetic acid, LFT: Liver Function Test, QC: Quality Control, EQAS: External Quality Assessment Scheme, ELISA: Enzyme Linked Immuno Sorbant Assay, PHC: Primary Health Centre, RFT: Renal Function test, CRP: C-Reactive Protein, TFT: Thyroid function test, TIBC: Total Iron Biding Capacity, PTH: parathyroid hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, GH: Growth Hormone, IGF-1: RBP: Retinol binding protein, AAS: Atomic Absorption Spectrophotometer, CV: Coefficient of Variation, HDL: High density lipoprotein cholesterol

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