Abstract

Biomarker studies are in great of interest in the field of pulmonary research and in this article, we reviewed the basic principles to collect biospecimens from the patients including blood, urine, induced sputum, bronchoalveolar lavage; how to handle them before extracting cells, RNAs, proteins and other compartments; hand how to store these biomaterials for the delayed analysis. We further summarized the types of study designs and ethical issues needed to be taken care of before starting a research for the researchers in the field.

Keywords: Biological Material; Biomarker Studies; Pulmonary Research

Introduction

It is very important to know “how-to's” before starting any research, and biomarker studies come in the front line of these, due the priceless materials used in the experiments: patients’ biological samples. It is very important to handle those biospecimens with care and store without losing any of the material of the consented patients, otherwise there may not be a second chance to obtain them. This article will make recommendations for the researchers who are interested in studying with the listed materials.
Obtaining and Handling Biological Material for Biomarker Studies

Blood and urine samples

Sample collection and storage prior to the analysis of biomarkers require careful handling. It is very important to work with care in each of these steps to obtain accurate and meaningful results from testing. In all well-known laboratories, how to obtain, transport and process each sample is determined by rules and all work is following these rules. The Clinical Laboratory Improvement Amendments Act of 1988 [CLIA 88] set and published these rules [1].

Obtaining whole blood/serum/plasma

The most common site for blood sampling is brachial vein in antecubital fossa of the arm. The first thing to do while blood sampling is application of a tourniquet proximal to the region of intervention. Thus, venous blood flow of the arm is interrupted, and the expansion and fullness of arm veins is achieved. Then, we determine the vein to puncture, it must be cleaned with 70% isopropyl alcohol and allowed to dry. After we get blood sample via a syringe or venous adapter, we transfer the sample into appropriate tubes. The size of these tubes may vary. Blood samples should not be received from site of intravenous [iv] treatment or veins located close to this site. However, if there is iv treatment from both arms, then we can get blood sample from a vein more distal to the iv catheter or directly from that catheter after cessation of treatment for at least 3 minutes. If the sample will be taken from the catheter directly, the first 5 ml of blood should be discarded as it may be contaminated with iv fluids [2-4].

In blood analysis, whole blood, serum or plasma can be used. When analysing “whole blood”, both the liquid portion of the blood called plasma and the cellular components of the blood [erythrocytes, leukocytes, platelets] are used. If the analysis will be done with whole blood, the sample must be taken into a tube containing anticoagulant and should be shaken gently for a few times in order to mix with anticoagulant thoroughly [7]. Thus, blood clotting in the tube is prevented. If this tube can wait or centrifuged, cellular components of blood precipitate to the bottom of the tube and leave a transparent yellow layer on the top. This yellow layer is called “plasma”. If we don’t add any anticoagulant into the tube, a fibrin clot containing blood cells occurs. When we allowed this tube to wait or centrifuged, there will be a clear yellow liquid layer at the top called “serum”. Most of the biochemical tests is performed using plasma or serum. The biggest difference between plasma and serum is plasma’s having anticoagulant and serum’s not having fibrinogen. Also, serum has higher levels of potassium. In obtaining serum sample, the whole blood should be allowed to wait for 30 - 60 minutes approximately in the room temperature in order to clot completely [5-7].

Caps of the tubes have different colors according to the additive inside the tubes. For example, tubes with purple cap contain ethylenediaminetetraacetic acid [EDTA] as the anticoagulant, while there is heparin in tubes with green cap. Heparin is also found naturally in the body, so its influence on test results is less except the coagulation tests. For the purpose of increasing the coagulation, tubes with red/yellow cap contain silica in the tube. These tubes should be gently shaken after adding blood sample into it to ensure mixing with the additives thoroughly. If you shake the tube so vigorously, this may lead to severe hemolysis and affect the results of studies with potassium, magnesium, lactate dehydrogenase, and similar enzymes [5-7].

Separation of plasma and cellular components is accelerated by centrifugation. For this purpose, the sample is centrifuged with a 1000-2000 x g relative centrifugal force (RCF) for 10 minutes. The “g” is gravity and $g = 9.81 \text{ m/s}^2$. We must pay attention not to cause hemolysis while centrifuging the sample. Following centrifugation, there will be ongoing metabolism among separate layers of cellular components and plasma/serum. In order to prevent the effect of ongoing metabolism on the test results, the blood sample can be put into tubes containing gel at the base. This gel settles between the plasma/serum and the cellular layer after centrifugation and prevents interaction between these two layers [6,7].

Transport of samples

According to CLSI and the Occupational Health and Safety Administration (OHSA) guidelines, blood samples must be transported to the laboratory in plastic bags with biohazard logo [6,7]. Transport via pneumatic tube system must be done via zippered or sealed plastic bags to prevent leakage or scattering of samples, and to reduce the potential effect of hits. Tubes must be transported with caps closed and carried upright. So, the movement decreases. That leads to better blood coagulation and prevention of safety risks caused by spilled
Obtaining and Handling Biological Material for Biomarker Studies

Obtaining and storing urine sample

Urine is the second most used sample after the blood. Urine sample can be obtained as random, the first urine in the morning, midstream urine sample, or urine is collected over a given period. For the quantitative measurements in urine, it must be collected for a period. This period is usually 24 hours. However, during outpatient follow-up, urine collection process is not fully carried out usually. In order to understand whether the urine sample is really a sample representing 24 hours, we can look to total creatinine amount in the urine. Creatinine excretion does not change significantly among people, and it is approximately 1 - 2gr for 24 hours in an adult. Although it varies from person to person, the average amount of daily urine in an adult is around 2 liters. The creatinine evaluated here is different from creatine clearance calculated to assess the kidney function [6].

After obtaining urine sample, a suitable preservative is added into it. For example, if we measure urine calcium, an acid preservative must be added to prevent precipitation of calcium phosphate. If long-term storage is needed, sample stability can be maintained by storing at -20°C or lower temperatures. Also, storing urine at -80°C is a common practice. However, it is not known whether such low temperature provides an additional benefit.

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**Induced sputum**

Neutrophil originate DNA, filamentous actin, microorganisms and cell remnants in the area contribute to the mucus secretion in the lungs [8]. Therefore, sputum examination is a non-invasive method that can provide information about components and severity of the diseases.

**Methods**

**Environmental preparation**

Jet or ultrasonic nebulizers can be used effectively for the solutions in sputum induction. To increase efficiency, output needs to be 1m/min if ultrasonic nebulizers are going to be used [9-11].

Spirometry is useful to evaluate the basal airway caliber and to predict airway hyper-reactivity with saline inhalation. If the patient has hypoxemia, oxygen supplement may be required, therefore oxygen saturation must be followed up and complete resuscitation equipment and personnel must be present throughout the procedure. Solutions that will be used in the procedure needs to be prepared fresh, bronchodilator agents [inhaled or nebulized salbutamol, ipratropium bromide etc] and emergency medications that may be needed should be complete. Moreover, infection control measures needs to be taken.

**Bronchodilation before the procedure**

Hypertonic saline nebulization may lead to bronchoconstriction in asthmatic subjects with a mechanism proposed to be related to mast cell and neuronal activation [8]. Therefore, guidelines on the topic recommend, application of short acting β agonists before the procedure [9,11]. Death has been reported in asthmatic people due to inhalation of pure water. Therefore, this should not be looked over. Salbutamol is usually administered once at a dose of 200 - 400 mcg, 2 - 4 puff via metered dose inhaler or 2.5 - 5 mg nebulized [12]. Salbutamol at higher doses have not been proven to be more efficacious to prevent hypertonic saline inhalation related bronchoconstriction and may result in more severe and less reversible bronchoconstriction at a later stage. Bronchodilator administration before or 10 minutes after spirometry is well tolerated and safe in children [11,12]. Bronchodilator administration before the procedure does not change cell distribution or number of the induced sputum [10,14].

**Monitoring the pulmonary functions**

Pulmonary functions are recommended to be monitored at intervals of 5 - 10 minutes or when symptoms appear during sputum induction. Most sensitive parameters for bronchoconstriction are forced expiratory volume at one second (FEV1) and peak flows [10]. Therefore, FEV1 is measured at first minute of nebulization and monitored at intervals of five minutes. If post-bronchodilator FEV1 change is less than 10%, then one measurement is adequate. Sputum induction may lead to a decrease in FEV1 to less than 65% of predicted. Repeated hypertonic saline may be applied if FEV1 is more than 80% of predicted. Cough and sputum expectoration are expected after each hypertonic saline nebulization and side effects are recorded if observed. Test is terminated if respiratory distress occurs, FEV1 falls below 80%, patient can’t be persuaded to finish the test or sputum can’t be obtained after 20 minutes even if FEV1 is above 80%.

**Preparation and application of the saline solution**

Saline solutions at concentrations of 0.9% to 7% can be applied via the inhalation route for two to seven minutes in sputum induction procedure. Saline concentration and nebulization equipment influence the tolerability and safety of the procedure; hypertonic saline can induce bronchospasm though it is less efficient than saline. Additionally, saline concentrations were proposed to influence mediators in the sputum, but evidence is not adequate [15,16]. For sputum induction, NaCl solution at a concentration of 4.5% can be used for each nebulization or the procedure can be started with NaCl solution at a concentration of 3% and increased to 4% and 5% at each inhalation (Figure 1). Asthma control needs to be considered when deciding the saline concentration for inhalation in asthmatic patients. In mild-moderate asthma, increasing concentrations of 3% -4.5% -5% can be used at five minutes intervals for one, four and five minutes.
In high risk asthma (severe asthma, reactive airway, acute exacerbation, use of high dose β2 agonists), inhalation is started with 0.9% NaCl solution and continued with increasing concentrations (3%, 4.5% saline can be applied at intervals of five minutes for 30 seconds, one, two, four minutes). Although hypertonic saline concentrations and durations vary between different studies, the resultant induced sputum has been shown to reflect airway inflammation [12,17].

**Figure 1:** Diagram shows algorithm to induce sputum to study biomarkers.

**Duration of inhalation**

Duration of inhalation influences the resultant sputum. At early phases of induction (0 - 4 minutes), neutrophils and eosinophils are prominent in the sputum then at later phases (16 - 20 minutes), number of lymphocytes and macrophages increase. Moreover, mucus concentration is higher in early samples while surfactant proportion increases later. Sputum samples obtained at early phases demonstrate central airways while samples obtained later reflect peripheral airways. Therefore, some researchers suggest early samples need to be excluded and late samples be analyzed [10]. Sputum induction can be repeated after 24 - 48 hours.

Spontaneous sputum has similar inflammatory cell and mediator content with induced sputum. However, cellular viability in spontaneous sputum is less than in induced sputum and the quality of cells is worse [18].

**Evaluation of the sputum**

Samples obtained with sputum inductions should be examined immediately; should not be stored more than a couple of hours at +4°C. To decrease contamination with saliva, samples should be taken from areas dense in sputum. Sputum is transferred into a tube after the tube is weighed; then weighing procedure is repeated to calculate the weight of sputum. It is then incubated with Dithiothreitol [DDT] solution at a concentration of 0.1% that opens the disulfide bonds that provides the gel form by binding the glycoprotein fibers, resulting in mucolysis. The sample that is obtained is mixed with equal amounts of ‘Dulbecco’s phosphate buffered saline’ D-PBS, shaked, filtered and centrifuged for ten minutes. Remaining sediment is mixed with PBS and the liquid phase components are measured. Total cell numbers are counted with Neuber hemocytometry and viability is evaluated. Cell mixture is prepared at a concentration of 1*10^6 cell/ml and centrifuged. When it is dried in the air, it is stained with Wright and cell distribution is examined. Metachromatic cell number is counted after fixation with Carnoy and staining with toluidine [19].

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If cell viability is < 50% and squamous contamination is > 20%, cell count is not reliable. Total cell number and cell distribution can be measured in the sample obtained with sputum induction while many mediators can be measured in the supernatant part with flow cytometry and immunohistochemical analysis. In healthy individuals, cells in induced sputum are composed of 60 - 65% macrophages, 35 - 40% neutrophils, 0.3 - 0.5% lymphocytes and epithelial cells. Eosinophilia in sputum is observed in uncontrolled asthma and chronic obstructive lung diseases; neutrophilia in infections, smoking and steroid resistant asthma [19-20].

**Diagnostic properties of induced sputum**

Induced sputum is a noninvasive method that has been used in evaluation of lung diseases and airway inflammation for a long time. In recent years, it has become an alternative to bronchoscopy and bronchoalveolar lavage (BAL) with development of qualified and dependable methods to obtain sputum. Cellular examination of sputum demonstrates the presence of inflammation and the neutrophilic or eosinophilic nature of this inflammation. Although, symptoms and lung function tests are used in chronic disease follow up, these do not reflect the inflammation. Determination of the inflammatory process in the sputum sample obtained by induction, especially in diseases such as asthma and COPD in which inflammation is the central pathogenetic mechanism, will contribute to better disease control. Recent evidence suggests that subjects with asthma and COPD have a smaller number of exacerbations and hospital presentations when their treatment is modified according to the inflammatory cell profile of their induced sputum [21,22].

Induced sputum studies about many respiratory diseases with various severity levels have demonstrated the relationship between airway function and inflammation, thus development of new disease phenotypes [20]. Determination of these different clinical statuses will enable better disease management strategies. Noninvasive induced sputum method has an important role in attainment of this end point.

**Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) fluid is another important biological material for pulmonary diseases and both the cells, and the fluid can be used for several purposes in pulmonary medicine. The algorithm for BAL fluid research is given in figure 2 and the recommendations to obtain BAL is given in table 1.

![Figure 2: Flowchart shows techniques and materials to study in the BAL fluid compartments.](image-url)
Identification and counting of cellular elements in BAL is an important component of BAL fluid assessment. Specific markers for bronchial and alveolar tissue may help in determining any contamination [23]. Cell analysis of the alveolar component should not contain squamous epithelial cells and bronchial epithelial cells should also be rare. If BAL contains more than 2% epithelial cells, it is likely to be contaminated with bronchial material. Lactoferrin and immunoglobulin (Ig) A are secretory proteins of the bronchial tree and should not be present in the alveoli. Their presence in BAL indicates bronchial contamination [23,24]. The number of cells in a non-smoker health individual’s BAL is given in table 2.

<table>
<thead>
<tr>
<th>Total number of cells &lt; 13,000,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell viability &gt; % 85</td>
</tr>
<tr>
<td>Differential cytology</td>
</tr>
<tr>
<td>Macrophage &gt; % 85</td>
</tr>
<tr>
<td>Lymphocyte ≤ % 10 - 15 [CD4/CD8 = 0.9-2.5]</td>
</tr>
<tr>
<td>Granulocyte ≤ % 5</td>
</tr>
<tr>
<td>Neutrophil ≤ % 3</td>
</tr>
<tr>
<td>Eosinophil ≤ % 1</td>
</tr>
<tr>
<td>Mast cells ≤ % 0.5</td>
</tr>
<tr>
<td>Plasma cells &lt; % 0.5</td>
</tr>
<tr>
<td>Squamous epithelial*/ciliary columnar epithelial cell** &lt; % 5</td>
</tr>
</tbody>
</table>

*Presence of squamous epithelial cells indicates contamination of upper airway secretions. **Epithelial cells > %5 indicates suboptimal sampling.

The first condition of a successful cell analysis is to maintain cell viability in the collected fluid. The saline used for BAL offers limited time for the protection of the cells (less than 1 hour) since it has low pH and nutrients. If the sample cannot be analyzed immediately, it is preferred to transfer it to tissue culture media (25 mM HEPES buffer with MEM or RPMI 1640) to form a supplement, for sample stability.
and store at 4°C. If the evaluation of the BAL sample is delayed for more than 24 hours, it is generally not considered suitable for analysis, even if the sample is in the form of a supplement.

**Storage of BAL fluid for later cellular analysis**

Both cellular and non-cellular components of the BAL sample can be frozen and stored if the immediate evaluation of BAL sample is not possible. After the centrifugation at 500g for 10 minutes and the supernatant is removed, the cell pellet can be stored in Hank’s balanced salt solution or in another tissue culture media (without Ca++ and Mg++), for morphological and functional studies [25]. In the ERS guideline, it is stated that the BAL sample can be stored at -20°C for 3 months, but if longer storage is needed, it should be stored at -70°C for enzyme activity and protein integrity [26].

Cells can be stored at 4°C for 24 hours without change in total cell count for subsequent analysis [27]. However, the onset of neutrophil apoptosis in 9 hours, the rapid phagocytosis of these cells by macrophages and because some proteins are heat sensitive, the BAL fluid should be frozen at -80°C until analyzed [28].

BAL samples can be stored for microbiological culture at 4°C for up to 24 hours without any loose of diagnostic value. However, after storage at -80° the culture has low reliability, especially for gram-negative bacteria [29,30].

**Electronic nose**

Human nose can discriminate various scents at low concentrations. Many research today investigates models that mimic the olfactory system. Electronic nose is a system that mimics human nose [31]. The two main components ate the chemical sensor and the analysis system. Not only can it sense and recognize a smell but also can detect the density of the smell and the family it belongs to due to the sensitivity of the sensor system [32]. Sensitivity is much higher than human nose due to the technology and may provide information about the concentration at standard measures.

Electronic nose has been developed using a system like the olfactory system in mammalian brain. Volatile organic compounds are caught by and attached to olfactory epithelium sensory neurons (receptors) in the mammalian nose. The signal that forms thereafter is transferred to the olfactory center and recognition pursues due to learning with education in the olfactory center of the brain. If the stimulus is a signal that has been sensed before and is known, then recognition happens in the brain. If it is a stimulus that is encountered for the first time, it is recorded in the brain as the “first”.

In the electronic nose, chemical sensors replace olfactory receptors. Scent molecules are sent to the chemical sensors through various methods. Contact of the molecules with sensor surface leads to changes in conductivity, resistance and frequency [32]. Signals picked up by the sensors from the environment are transferred into codes by the electronic systems and sent to the computer. Smell recognition pursues after these electrical signals are processed with various pattern recognition methods [33]. If this scent is encountered for the first time, the signal reactions that develop are recorded for this scent and the system recognizes this scent upon second encounter.

In many respiratory diseases, exhaled breath samples provide important information that can contribute to conventional diagnostic methods. Biomarkers in exhaled breath samples can provide information about inflammation, metabolic changes and the cells in the environment. Use of electronic nose system in examination of these samples may guide diagnosis and follow up of many respiratory and systemic diseases [34,35].

It has been reported that electronic nose can identify microorganism colonization of the airway in COPD patients that are clinically controlled. This data can contribute to follow up and prognostic estimation with the use of noninvasive methods in these patients [34].
Malignant diseases are another group in which electronic nose systems are of interest in the respiratory system. It is proposed that, detection of the defined biomarkers in exhaled breath with electronic nose may contribute to screening programs [36,37]. Sensitivity of electronic nose in lung cancer patients has been demonstrated as 71.4% while specificity is 91.9% [38].

Researches on subjects with asthma suggest that electronic nose may be used in determination of asthma severity and control [39]. Advances in diagnostic modalities aim to decrease invasive procedures, cost and to prevent side effects. Electronic nose technology is a non-invasive, rapid and relatively cheap novel diagnostic tool. Mobility and rapid results as well as field use without the need for hospital infrastructure increases the importance in medical field.

**Ethical issues for biomarker studies**

Biomarkers collected from human specimens are used to indicate exposure, disease or susceptibility. For instance, HLA-DPB1-Glu69 biomarker of genetic susceptibility is used to identify chronic pulmonary beryllium disease [40]. This would diminish the proportion of cases if those at risk were subsequently removed from the workplace, either by their own decision or those of management. Another instance is the examination of workers exposed to respiratory irritants for alpha1-antitrypsin deficiency who are susceptible to chronic obstructive lung disease [41].

Ethical issues in clinical research are based on the four principles: beneficence, non-maleficence, justice and respect for autonomy, also known as the Georgetown paradigm [42]. Beauchamp and Childress have suggested in addition four rules to guide ethical practice: veracity, privacy, confidentiality and fidelity [43]. New trends in ethics toward reciprocity, mutuality, solidarity and citizenship are described by Knoppers and Chadwick and fit better with environmental health concerns focusing on the health of the entire population, rather than individuals’ [44].

**Study design**

To discuss the ethical concerns, it is useful to describe the three temporal types of study design: contemporary, future and retrospective studies [45,46]. In contemporary studies, human specimens are collected, assayed and interpreted within a relatively short period. In contrast, future studies are designed for a long period, either targeted or open ended. In a targeted study design, human specimens are stored or banked for years; however, the actual study purpose is well described. In an open-ended study design, the priority is to achieve a unique source, and research projects will be determined years after actual human specimen collection. Retrospective studies have the characteristics of both contemporary and future studies. They use already stored and banked human specimens once collected for other research purposes. For instance, the JANUS bank in Norway has been collecting blood specimens for cancer research since 1973 [45,47]. Human specimens were collected and assayed for a certain biomarker and then all subjects were traced for their health status. However, the study results are today also used for other research purposes.

**Subject recruitment and informed consent**

Subjects can be recruited in many ways that can have ethical implications [45]. Investigators should be very careful not to force the subjects into participating in a study or are given false expectations. Informed consent is an authorization of an individual regarding his (her) participation in a study. It is not clear about the extent the investigator must go to inform the subject about the research protocol. However, investigators should inform the subjects of the risks and benefits of participation; detail the study activities and describe mainly any possible use of data in the future. At present, there is not a standard practice for the details required in informed consent documents. Investigators should notice that each subject truly understands the implications of participating in a study and that no questions remain.

**Privacy and confidentiality**

The subject does not consent to dissemination of the data that identifies him individually. The use of results beyond the purposes for which the human specimens were collected intrudes on subjects’ privacy. Inadvertent labeling of a subject as ‘abnormal’ can have poten-
Obtaining and Handling Biological Material for Biomarker Studies

Partially deleterious impact on the persons' ability to obtain insurance, a job or credit. Investigators should be aware that it also can affect the person socially [46]. However, in certain circumstances, such as when records are necessary to protect the health and safety of other persons; a court orders the records; needing records for uses compatible with the purpose for which the information was collected, etc. records can be disclosed.

Data protection framework

The use of biomarkers is controlled by domestic law and regulations [47]. Data protection framework includes; informing and asking participants' consent; collecting only the information for the identified purpose; retaining the information only as long as necessary; ensure that information is accurate and complete; to keep information secure; transparency in policies and practices; access of participants to their information [48]. Conventions and guidelines also rule the ethical aspects of human biomarker research. In Europe the convention on human rights and biomedicine is referred to as the 'Oviedo Convention' 1997; and 'Additional Protocol' 2005. Accordingly, human specimens can only be used if; no alternative of comparable effectiveness; no risks and burdens to its potential benefits including physical, psychological and social impact; scientifically justified research and accepted criteria of scientific quality; informed consent of study participants; right of study participants to information on the purpose, overall plan, possible risks and benefits and results of the research; and approval by the ethics committee.

Interpretation and notification of study results

Investigators should be cautious interpreting biomarkers. Inherent variability among subjects can influence the interpretation of the biomarker data [49]. Natural variability makes it essential to know the range of biomarker values in a normal population. For instance, normal cholinesterase levels in subjects exposed to organophosphorus insecticides may indicate a health risk for others. Investigators pretend that interpreting biomarker data being independent from social and political context. For instance, workers or community residents near a hazardous chemical source cannot be separated from use of such data [50]. Subjects may require recommendations on what to do about the study results, such as obtaining medical screening or surveillance, looking environmental changes to avoid further exposures.

The use of biomarkers reveals ethical considerations concerning study design; subject recruitment and informed consent; interpretation and notification of study results; maintenance individuals' privacy and the confidentiality [50]. There remain many questions including the ownership and access to specimens; whether specimens collected for one research purpose can be used for an entirely different research purpose; the balance between the individuals’ rights and common interest of potential benefit for the public or community. Investigators should be aware that biomarker information can affect the lives of subjects and their families without sufficient protection of personally identifiable data and the regulation of its use. The social power of biological information should be considered however, before any biomarker data are collected or used.

Conclusion

Clinical, basic and translational research studies have a great attraction to discover new biomarkers and possible therapeutic targets to deal with pulmonary diseases due to their beneficial effects in screening, differential diagnosis, determination of prognosis, prediction of response to treatment, and monitoring of progression of disease. To receive reliable results, techniques for obtaining and handling biologic specimens used in biomarker studies and the ethical issues should be reviewed carefully by the all researchers as well as clinicians.

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