BACKROUND

In August 1999, we conducted a field analysis of the feasibility of conducting benzene epidemiology studies in Shanghai, China. As a result of our findings, we concluded that Shanghai offers significant advantages for the conduct of concurrent and prospective molecular and clinical epidemiology studies on benzene hematotoxicity. The original charge of the investigative team was to assess the feasibility of benzene studies in China, including population-based case control studies and molecular epidemiology studies. Formal feasibility criteria were evaluated in this regard, and both types of study were deemed feasible. Prior to the visit it was not envisioned that benzene-induced disease would still be prevalent, that relatively high exposures still existed or that access to work sites and patient records could be easily and reliably obtained. Our subsequent studies and publications of results on benzene exposure in Shanghai, China, indicate that it is feasible to continue and bring to a successful conclusion concurrent and prospective molecular and clinical epidemiology studies of benzene toxicity in Shanghai, China.

Occupational Exposure to Benzene in Shanghai

Shanghai possesses an industry base that still experiences relatively high benzene exposure. This contrasts with recent NCI reports that conclude cumulative occupational exposure to benzene in China is generally less than 10 ppm/yr [1;2]. Several industrial segments continue to conduct operations that result in high benzene exposure for some workers, and some of these industries have operations essentially free of complex or confounding occupational exposures. Many of these factories also have long-term and detailed health and environmental records. A recent review of the 1999 benzene monitoring databases of the Shanghai Municipal Centre for Disease Control and Prevention (SMCDCP) and the Institute for Public Health Supervision (IPHS) revealed some factories in which area airborne benzene concentrations could be expected to result in biological effects in exposed workers. These results comport with personal observations we made during our visit to some of these same facilities in August of 1999. A follow-up analysis of SMCDCP monitoring records revealed 30 factories with measurements exceeding 12.5 ppm in 2000. Spot measurements conducted using a seletive benzene monitor confirmed some exposures in the range of 30-140 ppm. Results we have obtained and published over the past 5 years reveal significant occupational exposure to benzene for certain workers at even higher levels. Chinese authorities are acting to remediate benzene exposure conditions in a wide range of industries, and the situation with respect to occupational exposure to benzene is in a state of change.

The Shanghai Study Environment

The Shanghai area has a population base of approximately 23 million people. The prevalence of new cases of hematopoietic disorders presenting annually at Shanghai hospitals generally

exceeds those originally predicted at the beginning of this study: (e.g. Aplastic anemia (AA): 50; Myelodysplastic syndrome (MDS) : 60; and Acute myelogenous leukemia (AML): 120. In addition, there are additional cases of mild AA and MDS that are seen in central hospitals as outpatients.

Collaborating Organizations

- Fudan University

Fudan University (including the former Shanghai First Medical University) is a major university that is ranked in the topmost tier of Chinese national universities and health institutions. The Institutes of Biomedical Sciences is a leader in biomedical and health care research and has a distinguished faculty of Chinese and international scientists. It's president, Fuchu Hu, is a distinguished scientist and member of the Chinese Academy of Sciences. The International Clinical and Molecular Research Center (ICMRC) was recently established to provide a strong and continuing base for coordination of field studies and JCML clinical laboratory operations. Fudan University and its sister institutions, including Hua Shan Hospital and Shanghai Tumor Hospital, as well as the SMCDCP and IPHS, the Shanghai Hematology and Pathology Societies and participating hospitals will continue to support and participate in the clinical and molecular studies outlined in this proposal.

- Cinpathogen, Inc.

Cinpathogen, Inc. is a US corporation registered in Deleware with headquarters in Boulder Colorado. Cinpathogen was established by Dr. Richard Irons to provide a Western interface for the International Clinical and Molecular Research Center in the Institutes of Biomedical Sciences at Fudan University. Through Cinpathogen Dr. Irons has entered into formal agreements with Fudan University to coordinate and manage the ICMRC as the Chinese entity responsible for the continuation and completion of the Shanghai Health Study. The long term mission of Cinpathogen is to support development of the ICMRC as a sustainable home for JCML operations so that it can continue to partner health care with state of the art research in China. The principal directors of Cinpathogen, Inc. are Professor Richard Irons and Professor Liming Bao.

- University of Colorado Health Sciences Center

The University of Colorado Health Sciences Center is a nationally and internationally recognized biomedical research institution encompassing the Schools of Medicine, Pharmacy, Nursing, Dentistry and a NCI-designated Comprehensive Cancer Center. Under the leadership of Dr. Richard Irons, The Molecular Toxicology and Environmental Health Sciences Program (MTEHS) in the School of Pharmacy has become nationally recognized for its research on the pathogenesis and mechanisms of leukemia and lymphoma. The institutions that have participated in and supported this project include: the MTEHS Program; the Department of Pharmaceutical Sciences, School of Pharmacy; the Comprehensive Cancer Center; the Department of Pathology, School of Medicine; and the Colorado Genetics Laboratory (CGL).

- Shanghai Municipal Centre for Disease Control and Prevention and the Institute of Public Health Supervision, Shanghai Occupational Health Department

The SMCDCP and IPHS are the municipal government regulatory entities that are charged with the monitoring and enforcement of occupational standards in Shanghai. They have cooperated with the SHS Exposure Assessment Team to conduct detailed studies of industrial exposure records for important sectors of the industrial community in Shanghai.

- ExxonMobil Biomedical Sciences, Inc.

ExxonMobil Biomedical Sciences, Inc. is a subsidiary of ExxonMobil Corporation. The group employs nearly 160 specialists in toxicology, epidemiology, industrial hygiene, environmental sciences, exposure and risk assessment, and quality assurance. It is primarily engaged in support to ExxonMobil Corporation, but is also charged with designing, conducting, and collaborating in research that is relevant to the petrochemical industry as a whole. The group has considerable expertise in benzene health effects, and authored a comprehensive benzene risk assessment that formed the basis for the EU's Existing Substances risk characterization on benzene. The group has a track record of publications on benzene health effects and exposure estimation. Thomas Armstrong is an industrial hygienist with over 20 years experience, including monitoring and estimating benzene exposure for prospective and retrospective exposure assessments, domestically and internationally. A. Robert Schnatter is Section Head of Epidemiology and has performed studies of benzene exposure in petroleum and other related environments throughout the world.

Strategy for Study Implementation

- Differential Diagnosis of Lympho-Hematopoietic Disease in Shanghai

The major teaching hospitals in Shanghai are each administered through different independent organizations. Each is different in organizational structure, academic vs. clinical priorities, history, and politics that impact on performance if not the actual standard of practice as related to differential diagnosis and reporting of disease. Therefore, if we relied on existing diagnoses from individual hospitals we would expect variability in differential diagnostic procedures that could impact significantly on the experimental design of the proposed studies. Similarly, core bone marrow biopsies may be obtained for staging of lymphomas at some institutions but not others. Moreover, cytogenetic analysis is only rarely performed in support of diagnosis and is not used in routine follow-up of diagnosed cases of hematopoietic disease. Each department and each disciplinary unit (i.e. hematology, pathology etc.) adopts its own standards and applies them somewhat independently. Fudan University Medical Center, Hua Shan Hospital and Shanghai Tumor Hospital have good capabilities and a strong interest in collaborative benzene research programs. These institutions are high quality organizations but do not possess routine high-throughput molecular pathology, molecular hematology or cytogenetic diagnostic facilities as are common in major clinical cancer study groups in the United States. Differential diagnosis of the diseases of interest is predominantly performed on the basis of morphology alone. Therefore, our

approach is to establish independent procedures and capabilities for the diagnosis of these diseases.

- Laboratory Development

The defining strategy that we adopted for integrating the Shanghai Health studies, collecting, managing and controlling laboratory data, and collating patient information was to establish a Joint Clinical and Molecular Laboratory (JCML) at Fudan University Medical Center in Shanghai. In doing so, we established the premier clinical research laboratory in China that is fully competent in hematology, molecular epidemiology and cytogenetics, and that can also support occupational studies. JCML operations have now been transferred to the ICMRC. The laboratory is staffed by Shanghai, Cinpathogen, University of Cincinnati and UCHSC personnel under the overall direction of the P.I., Richard Irons. Shanghai personnel have been trained both in Shanghai and Colorado in order to provide independent laboratory operational capability. Further information on training is provided in Section 27. Laboratory operations involve specimen collection, processing, immunophenotyping, histopathology, hematology as well as primary cytogenetic and molecular analysis of samples collected during these studies. Additional capabilities will include teleconferencing, image processing and data transmission, fax and E-mail, among the participating institutions and the Shanghai laboratory. This has enabled the direct integration of clinical diagnostic procedures with experimental analyses outlined in this proposal.

The laboratory supports a standardized set of clinical diagnostic and research procedures for the characterization of cases included in these studies and for the analysis of occupational and clinical samples. The laboratory also serves as a primary and reference diagnostic laboratory for participating hospitals and cases of benzene poisoning and hematopoietic disease referred from across China. This has ensured that a uniform set of parameters is evaluated and that study quality control is maintained. The Shanghai laboratory will operate according to College of American Pathologists (CAP) guidelines. This strategy maximizes the quality of sample collection, preservation, data and documentation of quality control and minimizes the impact of regulatory and political restrictions on the exportation of human blood from China. In addition, by mutual agreement, the laboratory will accept primary responsibility for support of differential diagnosis of lymphoid and hematopoietic diseases at participating referral hospitals for the duration of the study. Finally, it provides a basis for the transfer of technical capabilities, information and academic exchange that are held in high regard by our Chinese colleagues.

I. ANALYSIS OF DISEASE PROGRESSION FOR APLASTIC ANEMIA, MYELODYSPLASTIC SYNDROME, ACUTE MYELOGENOUS LEUKEMIA AND BENZENE POISONING IN SHANGHAI, CHINA

1 OBJECTIVES

The purpose of this project is to continue to identify and characterize cases of blood dyscrasias presenting at Shanghai hospitals and to compare them, with respect to clinical presentation,

phenotype, molecular characteristics, benzene exposure, genetic susceptibility and prognosis. In addition, subjects with previously diagnosed benzene poisoning referred by physicians outside Shanghai are characterized and followed as individual cases. The format for the proposed studies is a set of clinical series superimposed on a case-control design. This provides the resolution and flexibility to integrate detailed molecular and clinical characterizations of the pathogenesis of these diseases, together with an evaluation of potential confounding and interacting factors, within a structured statistical analysis of benzene exposure.

1.1 Specific Aims

1. Characterize and compare benzene dose-response patterns for aplastic anemia (AA), myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML).

2. Identify and characterize cases of benzene poisoning (BP), AA, MDS, BID and AML and study the pathogenesis of these diseases.

   Goals include:

   a. Characterize the rate of progression from BP to AA, BID or MDS to AML to the extent possible based on the length of follow-up.

   b. Ascertain whether benzene induced-AML can develop independent of BP, AA, BID or MDS to the extent possible based on the length of follow-up.

   c. Ascertain whether there are unique clinical features for benzene-induced diseases.

   d. Compare clinical and molecular features of benzene-induced with drug-induced diseases.

3. Provide the basis for continued evaluation of differences in prognosis for subtypes of BP, AA, MDS and AML.

Two ancillary specific aims are to:

4. Provide differential diagnosis of NHL and AML cases for an accompanying case-control study of AML and NHL.

5. Support clinical diagnosis and case management of subjects presenting in participating Shanghai hospitals or referred to us from outside Shanghai.

2 BACKGROUND AND RATIONALE

2.1 sMDS/sAML

Following the introduction of radiation and chemotherapy in the latter half of this century, it became apparent that persistent cytopenias and other blood dyscrasias including AML could result from the use of these agents in the treatment of other neoplasias. These observations, together with an emerging literature on benzene toxicity, contributed to the realization that agents with the potential to produce bone marrow toxicity could also produce preleukemic changes or myelodysplasias (MDS) and that these frequently preceded the onset of leukemia in patients developing AML secondary to drug or chemical exposure [3-8]. The functional alterations in growth and differentiation between MDS and AML find analogy in the progression of solid tissue tumors from metaplasia and dysplasia to carcinoma and are linked through molecular genetic studies [9;10]. These observations, together with common molecular genetic changes and the frequent progression of secondary MDS (s-MDS) to secondary AML (s-AML), has lead to the inescapable conclusion that MDS and AML represent stages in the progression of a single disease continuum that is frequently observed in chemical leukemogenesis [11;12].

A diverse set of observations argue persuasively that the ultimate clonal derivation of most cases of s-AML and s-MDS are hematopoietic progenitor cells restricted to the myeloid lineage [13-17]. The earliest functional alterations observed for bone marrow cells exposed to leukemogenic agents involve altered cytokine response. Altered regulation of clonogenic response to Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) features prominently in both human and murine myeloproliferative disorders and is a frequent early observation in the development of AML [18;19]. Repeated exposure of mice to benzene in vivo enhances GM-CSF response [20], and chronic exposure to high concentrations induces a persistent myeloproliferative disorder [21;22]. Moreover, the benzene metabolite, hydroquinone, selectively enhances clonogenic response to GM-CSF in murine and human bone marrow cells [23-25].

Deletions of all or part of chromosomes 5 or 7 are the earliest clonal alterations that have been detected in s-MDS/AML. A number of gene loci have been mapped to chromosome 7; however, their function and role in leukemogenesis remain largely unknown [26]. The deletions associated with 5q- in s-MDS/AML are usually interstitial without translocation of the deleted material [27;28]. The variability of the breakpoints, together with characterization of the critical region (5q31) is consistent with the deletion of a critical gene sequence, rather than juxtaposition of DNA sequences that may occur in chromosome translocation [28]. A cluster of genes involved in the regulation of hematopoiesis are located at q31 on chromosome 5. However, there is no evidence for homozygous deletion of any of these genes in AML, such as has been described for retinoblastoma in which the absence of one allele is followed by a second somatic mutation resulting in loss of both copies of the gene [29].

Cases of s-AML developing following alkylating chemotherapy typically involve M1, M2, M4, M6, but historically not M3 or M5 subtypes of AML, based on the FAB classification system [30;31]. In many such cases there is difficulty in distinguishing between MDS and AML on the basis of blast frequency. Cases of M7 secondary to alkylating therapy have been reported, although this subtype is not commonly found in either spontaneous or treatment-related cases. These studies establish a consistent pattern in which the development of s-AML is preceded by a period of "preleukemia" in 33 - 80% of the cases, and is accompanied by clonal cytogenetic abnormalities predominantly involving loss of all or part of chromosomes 5 or 7. On average the frequency of deletions or loss of 5 and 7 in studies of patients who develop MDS and/or AML after antineoplastic therapy range between 67% and 95% [28;32-36]. The same cytogenetic abnormalities occur much less frequently in de novo AML: in 660 de novo cases chromosomes 5 and 7 were

observed in 4.2 and 4.4% respectively and simultaneously in 3.2% [37;38]. Most patients presenting with 5q- exhibit a "preleukemic" phase prior to the onset of AML and typically present within 3-5 years of initial treatment with alkylating agents [39;40]. A similar pattern also has been observed in studies of AML patients occupationally exposed to benzene and/or solvents among which benzene is the only recognized leukemogen [41-46]. However, these studies are uniformly hampered by the lack of quantitative, specific or even credible exposure assessments for benzene. The typical latency for benzene-associated AML appears to range between 10 and 15 years [47-51].

Previous studies examining peripheral lymphocytes in benzene exposed workers have suggested that chronic exposure to benzene in high concentrations results in aneuploidy with a very high frequency of involvement of chromosome 7 and solid evidence for involvement of chromosome 8 as well [52;53]. The marked disparity in the frequency of involvement of chromosomes 5 and/or 7 between de novo and s-AML suggests the possibility of a biomarker for use in evaluating causation. In contrast, other nonrandom clonal chromosomal abnormalities, such as +8 or +21, are also increased in either exposed or non-exposed populations, depending on the study [41;42;44;54;55]. Based on these studies it is evident that chromosomes other than 5 or 7 can be involved in the pathogenesis of s-AML; however, only aberrations involving 5 or 7 appear useful as biomarkers in discriminating between de novo- and s-AML [40;56]. An important observation that calls into question the use of chromosome aberrations in peripheral lymphocytes as biomarkers of effect for benzene exposure is that the pattern of aberrations observed in human hematopoietic progenitor cells exposed to benzene metabolites varies qualitatively and quantitatively from those observed in human lymphocytes [57]. Whether this difference also exists in vivo has not been determined. However, because hematopoietic progenitor cells are the ultimate target cells in the development of MDS and AML answering this question is crucial to our understanding of the significance of chromosome aberrations occurring in peripheral lymphocytes in vivo.

A distinct pattern of s-AML is observed following treatment with topoisomerase II inhibitors, primarily those agents that result in stabilization of DNA-topoisomerase II complexes, such as etoposide and teniposide. In contrast to the features described for AML developing following treatment with alkylating agents, these tumors typically arise within weeks or months of treatment, frequently are M3, M4 or M5 subtypes and are associated with balanced chromosome translocations involving 11q23, 15;17 and 21q22 [58-61]. In a relatively small series, the use of the intercalating topoisomerase II inhibitors, doxorubicin and dactinomycin, when used in combination with alkylating agents and irradiation, also has been associated with s-AML with chromosomal abnormalities characteristic of epipodophyllotoxin-related AML [62]. Using an indirect methodology, Eastmond and coworkers observed that benzene metabolites inhibited topoisomerase II in vitro and hypothesized that benzene could therefore produce AML exhibiting the "topoisomerase-genotype/phenotype" [63-65]. Recently, using direct methodologies, we have demonstrated that benzene metabolites actually inhibit etoposide-induced DNA complex formation via a catalytic mechanism, suggesting that the formation of topoisomerase–type chromosome lesions by benzene is unlikely [66]. Again, however, whether in fact this pattern of AML is observed in a well defined benzene exposed population is not known. A complicating factor in the resolution of this issue is that M3 and M5 subtypes are prevalent subtypes in *de novo* AML. Therefore, resolution of whether or not benzene exposure is associated with the development of this type of AML requires definitive exposure assessment. In previous retrospective studies it has proved impossible to resolve mutually exclusive hypotheses, such as primary MDS developing in a metachronous manner in unexposed or low exposed individuals and sMDS arising secondary to exposure to low concentrations of benzene. Therefore, it is central to

our understanding of the relationship between benzene exposure and the etiology of leukemia and related diseases that the relationship between chromosome abnormalities and the neoplastic process be defined. Unique and specific abnormalities occur non-randomly in subtypes of hematologic disorders that correlate with cell lineage, etiology and alterations in gene expression. These reoccurring abnormalities are found in over half of the cases of hematopoietic neoplasms that are cytogenetically studied. Others have non-reoccurring complex chromosome changes or have a normal karyotype.

2.2 Aplastic Anemia

Chronic exposure to high concentrations of benzene has long been associated with clinical evidence of bone marrow suppression and is generally thought to lead to the development of AA. However, bona fide cases of AA attributed to benzene have not been documented in the West for almost a generation, and anecdotal descriptions of AA developing secondary to benzene exposure are virtually devoid of descriptions of bone marrow and are not particularly informative [67-72]. Early clinical reports together with experimental studies suggest that lymphocytopenia and thrombocytopenia may predominate in subacute benzene toxicity [73-79]. However, benzene-induced AA has never been characterized in the modern era according to presently accepted diagnostic criteria, and even the question of whether benzene exposure results in AA, MDS, or both cannot be directly confirmed from existing literature. The Shanghai metropolitan area has a population approaching 15 million people; local hospitals admit about 50 new cases of AA per year based on previous diagnostic criteria. The incidence of new cases of AA in China, which approaches 3/100,000, is roughly 6 times that observed in the West. The reasons for this are not immediately apparent, but our initial published studies indicate that many of these cases should be classified as MDS rather than AA. Moreover, either disease developing as a consequence of drug exposure can be divided into different types according to either associated cytogenetic abnormalities or different patterns of bone marrow suppression that meet diagnostic criteria for classification as AA (i.e. anemia, pancytopenia and a hypocellular bone marrow). It should be noted that there are no generally accepted criteria for differentiating between these patterns except in their prototypical extremes. At present, the utility of using cytogenetic abnormalities as a prognostic indicator in AA is not well understood. However, clonal cytogenetic aberrations have been reported to 1) reflect previous exposure to alkylating agents and 2) be predictive of transformation into MDS or AML [80;81]. Based on our observations in Shanghai to date, it is possible that presumptions with respect to the prevalence of AA versus MDS will need to be revisited in light of results obtained from these studies.

2.3 Benzene Poisoning

"Benzene Poisoning"(BP) has not been treated as a separate diagnostic entity distinct from AML, MDS or AA. However, we have characterized unique form of benzene induced displasia (BID) that presents with features that distinguish it from classical forms of AA or MDS. BP is legally defined in China as a compensable occupational disease with the following clinical and exposure criteria: (a) a white count < 4,000/mm^3 or a white count of between 4,000/mm^3 and 4,500/mm^3 and a platelet count of <80,000/mm^3, (b) work for at least six months in a factory with benzene exposure, and (c) exclusion of other causes of abnormal blood counts (See Section 8.2).

2.4 Health Significance

The findings of this research have already and are expected to continue to clarify many unresolved issues with respect to the nature of bone marrow suppression, AA, MDS or MDS/AML, associated with chronic benzene exposure. Some remaining questions include: Does benzene exposure lead to the pattern of sAML observed following alkylation therapy or can benzene exposure also result in AML developing as a consequence of balanced translocations, such as those frequently encountered in primary AML or those observed following treatment with Topoisomerase II inhibitors? Is AML developing secondary to benzene exposure a high dose phenomenon or can it occur at relatively low concentrations? Is there a sharp discontinuity in the dose-response curve as suggested in recent studies employing cumulative exposure as a metric [50;51;82]? Is the dose-response different for individual diseases, i.e. AA, MDS or AML? Can benzene-induced AML present in the absence of previous evidence of blood dyscrasias or sMDS, or is it virtually always a consequence of a progression from clinically significant bone marrow suppression to sMDS to sAML? Are there differences in the clinical presentation, response to treatment and risk of transformation to sAML between cases of idiopathic acquired AA and AA developing as a result of benzene exposure? Nearly all of these questions have a significant impact on determining prognosis for patient counseling and for future benzene risk assessment.

2.5 Research Design

The over-arching study design will be a case control study for: AA, AML and MDS (case-control comparisons for AML will also be provided in an independent NHL/AML case control study). This design is necessary to compare benzene dose-response for individual disease entities. In addition, case series descriptions and a comparison of selected parameters and disease progression will be conducted for AA, MDS and AML. Controls for the case-control study will be matched individuals presenting with non-lympho-hematopoietic cancer and controls for the case series will be cases of the same disease that are not likely to be due to benzene exposure (See section 8.1). Hematopoietic diseases with known heritable or nutritional etiology are specifically excluded (See section 8.B).

A Identification of the Study Populations and Enrollment of Subjects

Cases will be ascertained from five different sources, which will encompass various cases of disease severity. The five sources will consist of: (a) inpatients presenting at 30 primary and tertiary referral hospitals in Shanghai, (b) outpatients with mild disease presenting at district central hospitals in Shanghai, (c) cases of BP referred by rural workplace clinics, hospitals and physicians outside of the Shanghai metropolitan area, and (d) surviving cases of BP reported in the SMCDCP database since 1986. In addition, any cases of BP not identified through the above sources, but present in a companion molecular epidemiology study are also be eligible for recruitment as cases. The recruitment period for hematopoietic diseases will extend through June, 2007 and for lymphoid neoplasms through December, 2007. Specific clinical criteria for ascertainment of cases from these individual sources are outlined in the Research Protocol.

A.1 Inclusion Criteria

a Any individuals 18-75 years of age presenting at participating referral hospitals with any combination of signs or symptoms consistent with severe AA, MDS or AML are potential subjects for this study.

b Mild cases of AA or MDS presenting as outpatients at Central District hospitals will be referred to Hua Shan Hospital. If any of these cases present signs or symptoms consistent with AA, MDS they will be recruited to this study. Specific criteria for mild and severe disease are outlined in Section 13.

c A small number of "outside" cases of BP (or benzene-related AA, MDS or AML) referred by physicians from outside Shanghai will be recruited as separate cases.

d Surviving cases of BP identified from the SMCDCP benzene poisoning database will be recruited as part of the BP case series.

e In addition, a companion molecular epidemiology study may identify cases in advance of routine reporting procedures. These cases are also eligible for inclusion.

A.2 Exclusion Criteria

Patients in A.1.(a) presenting with heritable (See list of excluded conditions in 8.B below) or conditions not associated with drug or chemical hematotoxicity will be excluded from enrollment (e.g. evidence of nutritional deficiences: iron, vitamin B12, folate). Because comparison of the clinical and molecular characteristics of drug-induced disease with benzene-induced disease is a goal of the study, cases of disease developing secondary to chemotherapy or other agents are not specifically excluded.

A.3 Selection of Controls

For each hospital-based case of BP (referred from ME study), AA, MDS, BID or AML two matched hospital-based controls will be selected. Controls will exclude all diagnoses which have been hypothesized as linked to benzene exposure or that are within the inclusion criteria of this study. Controls will also exclude all other lympho-hematopoietic cancers but will include other neoplastic and non-neoplastic diseases. Specific excluded diagnoses are: non-AML leukemias, NHL, Hodgkin's disease, and multiple myeloma. Controls will be individuals presenting at the same institution, matched by gender and date of birth (+/- 5 years). These criteria may be modified in consultation with the external scientific review panel (SRP) The potential controls meeting the above criteria and admitted closest to the case's admission date will be chosen by study/hospital personnel and recruited to the study. Hospital-based controls will not be selected for cases of AA, MDS or AML referred to us from outside Shanghai. Controls will be subjected to the same benzene exposure assessment as study cases.

A.4 Informed Consent

Written informed consent will be obtained from all subjects in their native language at the time they are recruited to the study. Informed consent will be obtained directly by the study coordinators who will independently ensure that subjects comprehend the nature, benefits and risks of participation.

B Analysis of Exposure

Enrolled subjects' concurrent and previous medical history will be carefully evaluated for evidence of relevant infections or exposure to hematotoxic drugs or agents using a written checklist. Subjects' and controls' previous exposure to benzene will be classified according to duration of exposure, concentration of exposure (when possible), and exposure variability and intermittency (when possible). It is expected that for some cases, a ranking of exposure, possibly tied to concentration ranges (e.g. < 1 ppm, 1-10 ppm, etc.) will be feasible in the absence of necessary underlying data on precise exposure concentrations. Exposure assessment will be conducted blindly – i.e. without revealing whether a subject is a case or control. For the case subset comparisons, the "outcome of interest" will not only be benzene or confounding exposure, but clinical features of the disease (i.e. blood counts, phenotypic characteristics of the underlying bone marrow lesion, and clonal cytogenetic abnormalities). Available previous clinical laboratory data will be included as part of clinical history but will not be reported as study data.

C Sampling Strategy

C.1 Clinical Assessment

The bone marrow and/or blood of patients identified as candidates for enrollment in groups A.1(a-e) above will be obtained and analyzed for hematology, morphology, immunophenotype, and presence of cytogenetic or molecular lesions consistent with standard practice for the differential diagnosis of the diseases of interest. Bone marrow cells will be frozen for subsequent analysis of growth characteristics in culture. For potential cases of NHL that will be diagnosed in support of Specific Aim 4 (1.1), tumor biopsy tissue and blood will be analyzed. Tumor and bone marrow cells will be frozen for future culture and molecular analysis. Serum will be analyzed for previous exposure to infectious agents that pose a risk for NHL: i.e. HCV, as well as HHV8. Human immunodeficiency virus (HIV) is a risk factor for immunosuppression induced NHL. HIV will be measured as part of the study protocol and will be available in support of clinical diagnosis. Positive results will be reported to subjects' physician according to Chinese law. This fact will be included in subjects' informed consent forms. Cases of AA or MDS (A.1.b) that present as outpatients at Central District Hospitals will be referred to Hua Shan Hospital for recruitment to the DP project. For groups A.1.a, c-e, B lymphocytes will be immortalized and frozen for potential analyses of genetic polymorphisms of susceptibility and/or prognosis that are beyond the scope of the present study.

During the course of this study, cytogenetic analyses will be performed as part of diagnostic work-up on subject's samples including peripheral blood, bone marrow aspirates/bone marrow core biopsies, and tissues such as lymph nodes. According to initial clinical presentation, subjects will be either screened by FISH with a panel of DNA probes, or G-banded chromosome analysis

as an initial step and followed by appropriate FISH studies to further confirm findings from standard cytogenetics analysis. Cases with complex chromosome abnormalities will be further analyzed using either multiplex-FISH (M-FISH), a procedure using multiple color chromosome painting, or comparative genomic hybridization (CGH). These latter techniques will provide additional information with regard to chromosome aberrations and quantitative genome changes for diagnoses of patients with complex chromosome abnormalities.

C.2 Exposure Assessment

We will apply a tiered approach that provides flexibility to meet several study data needs. For the disease progression and case control studies, first tier assessments will be used for sorting cases and controls into likely unexposed, uncertain, and exposed categories. Then, more detailed tiers will be used to clarify the uncertain exposure category, to further quantitate the exposed categories, and to determine the time-patterns of exposure. The final exposure matrix will be developed after review and consideration of several data sources for exposure assessment including the IPHS data set, Yang Pu database, the Chinese literature, real-time monitoring in selected factories and simulations to arrive at credible benzene exposure estimates.

D Analysis of Confounders

Potential confounders and effect modifiers will be collected through the use of structured questionnaires. Effect modifiers are exposures, lifestyle factors, or host factors that can modify the effect of benzene exposure for each disease. Confounders are risk factors for the disease that may distort the exposure-response relationship through specific distributions in case vs. control, and high versus low exposure subgroups. Both effect modifiers and confounders, as described below, will be controlled in statistical analyses.

It is generally recognized that the majority of alkylating agents used in cancer chemotherapy are capable of producing MDS and/or AML, including: melphalan, chlorambucil, busulfan, cyclophosphamide and nitrosourea compounds such as carmustine (BCNU) [3;83]. Because of their frequent use in combined treatment regimens, it is often difficult to dissect out the respective roles of certain individual chemotherapeutic agents. For example, the individual role of cisplatin in the development of MDS or AML is uncertain, but its use in combination with doxorubicin or etoposide has been demonstrated to result in the development of AML [84]. Far less potent but possibly leukemogenic agents include the purine antimetabolite, azathioprine, as well as procarbazine, adriamycin and bleomycin [85-87]. The frequency of s-MDS/s-AML varies markedly depending on individual therapeutic regimen. For example, one study of patients treated for multiple myeloma reported an AML incidence of 2.6% for those treated daily with melphalan compared with 0.7% in those patients receiving single doses of melphalan, BCNU and cyclophosphamide. Overall, the incidence of s-MDS/AML following alkylating therapy has ranged between 0.6% and 17% with relative risks averaging about 100 fold (range 9-320 X). In contrast to primary cases of MDS such as that associated with the spontaneous 5q- syndrome, the rate of malignant transformation to AML in treatment-related MDS is remarkably high [36;88]. In addition, the epipodophyllotoxins, etoposide and teniposide, are also associated with high rates of s-AML, and, in a relatively small series, the use of the intercalating topoisomerase II inhibitors, doxorubicin and dactinomycin, when used in combination with alkylating agents and irradiation,

also has been associated with s-AML with chromosomal abnormalities characteristic of epipodophyllotoxin-related AML [62]. To the extent possible, we will characterize treatments, such as chemotherapy, that result in MDS/AML and control these effects in statistical analyses, so that the effects of benzene can be characterized independently.

There also has been considerable debate over whether cigarette smoking is a risk factor for the development of leukemia. Austin and Cole suggested that a link between cigarette smoking and leukemia might exist, concluding that experimental and epidemiologic evidence suggested that smoking was more likely related to AML rather than to other types of leukemia [89]. Since that time major inconsistencies have characterized most of the literature in this area. Studies have variously observed increased risk associated with smoking and AML [90-94] or alternatively no increased risk of leukemia. These inconsistencies are explained in part by the small relative risk (i.e. odds ratios of ~1.5 observed in most studies) and the difficulty in controlling for an exposure/behavior as prevalent as cigarette smoking in the general population. A recent meta-analysis suggests both an increased risk of myeloid leukemia/acute non-lymphocytic leukemia and a consistent dose response relationship between cigarette smoking and acute non-lymphocytic leukemia (AML) [94]. In support of biologic plausibility, most studies invoke low level contamination by known leukemogens, such as benzene and ionizing radiation, in cigarette smoke. When integrated over time, cumulative exposure to benzene from cigarette smoking is comparatively high [95;96]. Nevertheless, the absolute dose of benzene associated with the smoking of a cigarette is likely to be far lower than that demonstrated to produce any adverse affect in animals or humans. Even so, it must be said that the total dose of benzene metabolites (e.g. phenol, hydroquinone and catechol) present in cigarette smoke, independent of benzene, far exceeds that potentially derived from benzene itself [97;98]. Moreover, cigarette smoking is known to dramatically increase peripheral leukocyte counts and presumably may alter regulation of hematopoiesis independent of chronic low level exposure to these specific agents [99-102]. The observed relative risks of leukemia associated with cigarette smoking are low, usually between 1.0 and 2.0. Nevertheless, the prevalence of smoking in the general Chinese population, predominantly men, together with the possibility that cigarette smoking may alter hematopoiesis and indirectly influence the risk of leukemogenesis associated with other agents, warrants tracking of smoking history in this study. To the extent possible, we will characterize and control for cigarette smoking in all analyses.

Leukemias occurring as a consequence of high doses of γ- or X-ray were the first secondary neoplasms associated with survivors of the atom bombings of Nagasaki and Hiroshima. Following single exposure to high dose gamma radiation, the survivors experienced an elevated risk of AML, ALL and CML [103-105]. Leukemia excesses were limited to those exposed to greater than 10 Grey. A similar pattern of leukemia excess has been observed in individuals receiving high dose γ or X-ray therapy, or those receiving thorotrast, with a somewhat smaller relative risk observed in patients receiving radiation therapy alone than is observed following chemotherapy or combined radiation-chemotherapy [105]. The pattern of leukemias occurring secondary to γ-X-ray radiation or combined therapy differs from that observed following chemotherapy alone in that AML is the only leukemia type consistently observed in excess in patients receiving chemotherapy alone [106]. Studies on the relationship between low dose γ- and X-ray exposure, secondary to fallout or diagnostic X-ray, and leukemia incidence are confusing, controversial and difficult to interpret based on dose/response [105]. Nevertheless, occupational exposure to ionizing radiation is

to be considered an important potential confounder in these studies, and we will seek to control it to the extent possible.

Evidence of previous infection (tuberculosis, brucellosis, etc.), especially from viruses (e.g. Hepatitis C, Epstein-Barr) have been shown to be strong risk factors for certain lymphomas, and are suspected risk factors for AML/MDS. Previous medication use (e.g. chloramphenicol, sulfonamides, non-steroidal inflammatory agents) are also risk factors that will be controlled in all analyses. Host factors (age, gender) will be assessed as confounding factors and will be used in final logistic regression models (see below).

E Statistical Analysis

Statistical analyses will be appropriate for the different specific aims of the study. For dose response analyses, statistical analyses will initially employ traditional Mantel-Haenszel techniques for matched sets. [107]. Conditional odds ratios will be calculated over all matched sets for AA, MDS, and AML. STATA and SAS software packages will be used for these analyses. Quantitative exposure estimates will be categorized to assess exposure response. In addition, logistic regression analyses will be used to examine continuous benzene exposure metrics. In these models, case/control status is the dependent model variable. Independent variables will include quantitative benzene exposure metrics, and potentially confounding variables. As a general rule, potential confounders will be kept in the final model if its presence changes coefficients (odds ratios) by 25% or more. Model fit will also be evaluated.

For assessing disease progression, it is anticipated that formal statistical analyses (such as survival or life table techniques) will not be warranted. The question of whether one disease state is a prerequisite for a (subsequent) disease step can be disproven by observing the subsequent disease step in the absence of the hypothesized prerequisite. Conclusions regarding requisiteness are of course strengthened by observing the hypothesized prerequisite in a large case series. Simple binomial and Poisson probability theory can be used to assess the likelihood of observing two (or more) disease states together, when in fact, no relationship exists.

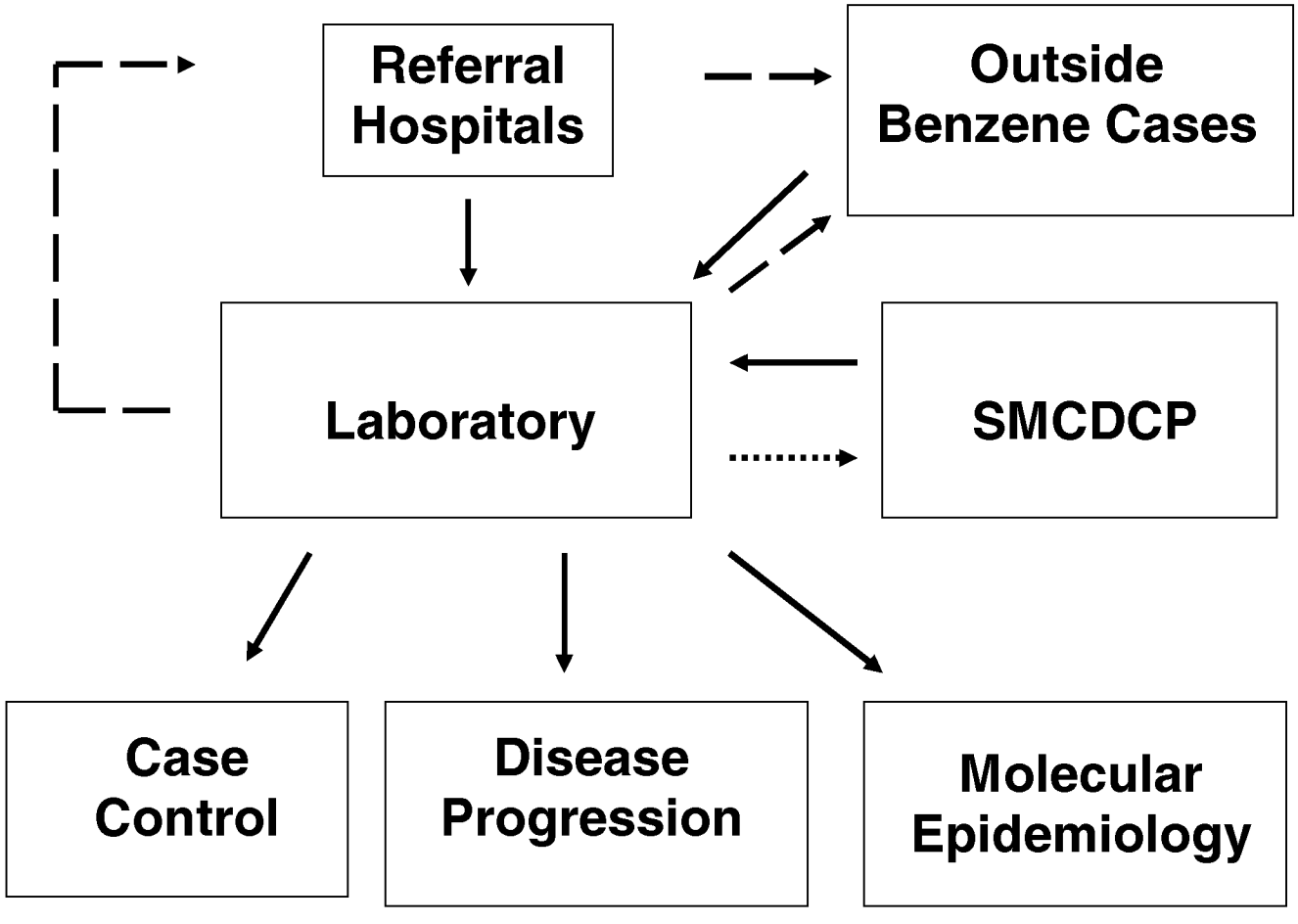
For assessing whether there are unique clinical features for heavily exposed patients, comparisons between case subsets will be made. Depending upon the background rate of the clinical lesion in question, either Poisson or binomial probabilities will be assumed. If the background rate (from the literature and historical clinical experience) is less than 5%, a Poisson probability distribution will be assumed. High exposure cases will be initially defined as definitively high benzene exposure for a number of years (e.g. >50 ppm for 5+ years). Non-exposed cases will be restricted to those where there is a relatively high degree of confidence that there was no more than background benzene exposure (e.g. no employment or hobbies that involved benzene). The presence of specific clonal cytogenetic structural aberrations (e.g. $5q^-$ 7q-, -5,-7, t(15;17), etc.) will be quantified for high versus no exposure cases and compared using chi-square statistics. Sensitivity analyses using alternate high exposure groupings will be performed.

F Organizational Aspects

Patients presenting at any one of the participating hospitals in Shanghai will be candidates for enrollment in the study. Additional subjects reported in the SMCDCP database to have been diagnosed with BP, newly diagnosed cases of BP presenting at Central District Hospitals or identified in the accompanying Molecular Epidemiology Study or BP cases independently referred by physicians outside Shanghai will also be asked to participate. Study coordinators will be staff hematologists selected by their respective institutions to participate in the study. They have been trained on protocol requirements and informed consent issues and assisted in interviewing and sampling individual patients by study personnel from the UCSHC-FUMC Joint Clinical and Molecular Laboratory and the ICMRC. All clinical analyses will be performed by the ICMRC in collaboration with the Department of Pathology, Shanghai Cancer Hospital, and the Department of Hematology, Hua Shan Hospital. Confirmation and consultation on histomorphologic, cytogenetic and molecular aspects of diagnosis is provided by participating UCHSC faculty and laboratories in Denver, Colorado. Exposure analyses will be performed by study personnel at Fudan and SMCDCP under the direction of Dr. Armstrong and Ms. Zhong. Training of ICMRC staff has taken place in Shanghai and Denver under the auspices of UCHSC. Modifications to the Specific Aims or significant changes in the scope of the study have and will continue be brought to the attention of the External Scientific Review Committee. Similarly, the Ethics Review Committee will be consulted during the conduct of the study when protocol design or ethical concerns arise that have not been previously addressed.

The laboratory receives patient information and samples from referral hospitals and from cases of BP referred to ICMRC/JCML from physicians outside Shanghai. Laboratory, exposure and questionnaire data obtained for cases recruited to each study will be transmitted to each appropriate study data base (Solid Arrows). ICMRC/JCML communicates clinical laboratory data to hospitals and physicians in support of diagnosis and case management (Dashed Arrows). SMCDCP databases for benzene monitoring and BP have been used and evaluated to identify individual factories for study in the Molecular Epidemiology Study (See Protocol II). Data collected as part of the exposure analysis, as well as relevant clinical findings and technical recommendations on remediation, are communicated to SMCDCP (Dotted Arrow).

Figure 2.1 Laboratory Organization



F.1 Cases of Severe AA, MDS, AML, BID or BP Cases

Subjects will be recruited to the study by hospital Clinical Coordinators on the basis of initial clinical presentation, clinical history and/or previous preliminary blood work. Interviewers will obtain informed consent, administer study questionnaire and schedule blood draw and bone marrow biopsy-aspiration procedure with coordinator, hospital and laboratory Collection Team. In addition, interviewers will provide Exposure Assessment Team with the subject's questionnaire information pertinent to identity, employment and exposure history. BP cases identified in the companion Molecular Epidemiology study will be admitted to a participating hospital, subjected to full benzene exposure assessment and assigned a control.

F.3 Outside cases of Benzene-induced AA, MDS or AML

A small number of cases of benzene-induced disease referred by physicians outside Shanghai will be recruited as cases. Criteria will include definitive (quantitative or anecdotal) evidence of previous benzene exposure and a diagnosis of BP. These cases will be subject to full benzene exposure analysis but will not be assigned hospital controls. Depending on individual circumstances, patients will be evaluated either in Shanghai or in their city or province of residence.

G Follow-up

The presenting clinical and molecular characteristics of individual cases of BP, AA, MDS and AML will be characterized and recorded in the study database. Cases of BP, AA and MDS also will be re-evaluated at regular 6 month follow-up periods through June, 2007. Results will be compared in order to evaluate the clinical and molecular progression of these diseases and will be reported to treating physicians. Cases of AML will not be subject to routine follow-up as part of this study but will be followed in support of clinical management at the request of the treating physician.

3 PROJECT MILESTONES AND DELIVERABLES

3.1 Deliverables

Case Control and Disease Progression Studies

- Continued laboratory operations and diagnosis of Hematopoietic and Lymphoid Neoplasms (WHO, 2001) for participating hospitals in Shanghai.
    - AML, MDS, AA, BID, BP through 6/30/07
    - BID, BP through 12/31/07
    - Continued case accrual for DP and diagnostic support through 12/31/07
    - NHL through 12/31/07

- Verified study diagnoses of approximately:
  - 500-600 cases of AML
  - 400-500 cases of NHL

- Follow-up of Disease Progression through 12/31/07

- Continued maintenance of the JCML Tissue Bank and formal transition of the Tissue Bank to ICMRC.

- Assessment of Benzene Exposure for above casesand a matched (2x) set of controls with documented IC's and questionnaire administration for all cases and controls.

- Review and consideration of several data sources for exposure assessment including the IPHS data set, Yang Pu database, the Chinese literature, real-time monitoring in selected factories and simulations to arrive at credible benzene exposure estimates.

- Development of an EA matrix using the above data sources for all study cases and controls.

- Continuation of QA procedures for documentation and cooperation with an independent QA auditor.

- Migration of data RE. diagnosis, confounders and EA to CC study coordinator.

Molecular Epidemiology Study

- Quantitative IH analysis of benzene, toluene, xylenes for workers in 5 factories (est. 500-750 workers) in which benzene is present.

- Continuation of the development and validation of methods for the analysis of benzene metabolites.

- Application of the above methodology to the analysis of a subset of workers in these 5 factories.

- Continuation of measurement of hematologic parameters and genetic polymorphisms for workers in these 5 factories

Post-Study Professional and Technical Support

- Final case diagnostic review and completion of CC and DP datasets

- Post-study data analysis and migration

- Continued data-analysis (ME, DP) preparation through June, 2008

- Submission of study publications (2 ME, 2DP)

A Publications

The principal product of this study shall be the generation of reports to be published in the peer-reviewed scientific literature. Manuscripts and abstracts prepared for this purpose will be submitted to the Scientific Review Panel for constructive advice and suggestions prior to submission for publication. US regulatory concerns about publishing industry-sponsored studies most often focus on the independence of study investigators. Reprints will be furnished to Study Sponsors after scientific review and acceptance for publication. Whenever possible, the PI will provide notification of publications to the Sponsors at least two weeks prior to publication. In any event, the final decision to edit or submit a manuscript for publication will reside exclusively with the Principal Investigator.

B Interim Reports

The Principal Investigator will submit interim progress reports at 6 month intervals to the SRP and Study Sponsors. These reports will characterize study progress, significant developments or difficulties as well as budgetary details. The inclusion of scientific findings in Annual Reports shall be at the discretion of the Principal Investigator, unless these findings are the subject of manuscripts published or accepted for publication. In addition the PI will submit interim monitoring reports on metrics for case accrual and EA progress various times as determined appropriate by the QA/QC Assessment Team.

4 BENEFITS

4.1 Contribution to the Worldwide Community

The proposed research will provide a detailed understanding of the pathogenesis of benzene-induced bone marrow injury and clarification of the specific identity and distinguishing characteristics of blood dyscrasias developing as a consequence of benzene exposure. This information can be used to enhance and refine efforts to protect human health. Exposure analysis will provide valuable information on the nature of the dose response for benzene-induced disease,

specifically addressing questions of threshold for benzene-induced hematologic diseases. Samples and materials collected during this study will provide the potential for future analysis of genetically determined susceptibility. This will permit the future identification of individuals with potentially increased risk of benzene-induced disease and provide a scientific basis for improving regulatory decisions aimed at preventing health risks associated with benzene exposure.

Independent of the study of benzene-induced disease, the proposed research will identify characteristics or biomarkers of the individual diseases under study that may serve as predictors of outcome or prognosis in individual patients.

4.2 Contribution to the Study Population Community

The development of the ICMRC laboratory, together with training of Shanghai personnel, will allow transfer of laboratory technology and expertise to the local community. Moreover, because of the limited amount of clinical sampling material available, ICMRC will collaborate with participating hospitals in providing routine clinical laboratory support for the diagnosis of lympho-hematopoietic disease. The integration of the ICMRC laboratory into the diagnostic paradigm of participating hospitals will also serve to enhance and improve the differential diagnosis and management of lympho-hematopoietic diseases in Shanghai.

4.3 Contribution to Individual Members of the Study Population

Integration of ICMRC into the diagnostic paradigm of participating hospitals will contribute directly to the benefit of individual patients by providing clinical laboratory support for the diagnosis of their disease. Further, the technologies employed by the laboratory will provide enhanced capabilities for differential diagnosis of individual patients than are currently available at participating institutions. These technologies enable follow-up and monitoring of disease status that is important in the management of individual patients and that is not possible with existing standards of diagnosis in Shanghai. During the conduct of the study the guiding philosophy will be place a priority on support of the clinical management of patients and study subjects. Laboratory findings of clinical importance will be communicated to referral physicians independent of their relevance to study objectives. The close working relationship between the ICMRC, IPHS, SMCDCP and other public health regulatory authorities will facilitate worker protection when BP cases are identified, significant hematologic abnormalities are found or unacceptable benzene exposure concentrations are encountered. Further, characterization and follow-up of individual cases of benzene poisoning will provide direct benefit to these subjects in the diagnosis and management of their disease.

5 SOCIAL IMPACTS AND ETHICAL IMPLICATIONS

5.1 Ethical Implications

The proposed study involves patient sampling and the use of biological specimens as well as the collection of personal data and genetic information. Therefore, the protocol has undergone review by an international Ethical Committee convened for this purpose, and chaired by Prof.

Baruch Brody of Baylor University. Further, informed consent forms and questionnaires, translated into Mandarin, were subject to Chinese ethical review in July, 2001 by Professor Xu Zong Liang of Fudan University and modified according to his recommendations, protocol ethical considerations will again be reviewed by the COMIRB and again by the Fudan University IRB, the SMCDCP IRB and Chinese government licensing authorities in Shanghai.

All study participants will be informed of the purpose of the study and will be asked to sign an informed consent form. The results of these studies will be communicated to local community regulatory and medical authorities, and the results of individual patient tests will be communicated to treating physicians. All study subjects will be compensated for participation in the study at levels reflecting the degree of inconvenience, the invasive procedures required and that are in keeping with local social and ethical standards of practice.

5.2 Patient/Subject Confidentiality

Subject confidentiality will be maintained at the level of analysis and dissemination of study data. The clinical laboratory activities in which JCML will be engaged require extremely high standards of reliability with respect to patient identification and communication of clinical data. Because it is impossible to blind laboratory personnel to patient identification, laboratory personnel will treat patient clinical information with appropriate professional standards of professional confidentiality. Access to the clinical database will be encryption-protected. A separate study database will be maintained at UCHSC for dissemination and analysis of study data. The study database will be networked with the clinical database at JCML but will be access-protected in Shanghai using firewall technology.

5.3 Subject Compensation

Determining appropriate compensation for subject participation in these studies is complicated by disparate cultural and economic issues between China and the United States. Patient/subject compensation in this study has been structured so as to not be so high that it provides an undue inducement but not so low as to exploit the subject population. The specific compensation schedule for participation of study subjects also should reflect Chinese cultural norms for participation in clinical research which are different from those typically encountered in the United States. For example, it is against Chinese law to directly link compensation to obtaining human tissue in any form. We used as a point of reference blood donation for transfusions in China which normally amount to less than half the blood volume (200 ml) donated in the West and for which the donor receives no money but gets two weeks paid leave. Across the breadth of the Chinese work force in cities such as Shanghai, average monthly wages vary between US$100 and $1000. However, wages in China and especially Shanghai are increasing at a rapid pace. Following consultation with Professor Xu, a paradigm for compensation was adopted:

Participation by control subjects providing only an interview will be reimbursed at US$30. Individuals asked to provide a blood sample via venipuncture (6-10 ml) will be compensated US$52.50 for the interview and blood draw. Subjects who provide an interview, blood via venipuncture and undergo bone marrow biopsy and aspiration will be reimbursed US$120. Subjects who provide an interview, blood via venipuncture and a tissue biopsy will be

compensated US$60. The latter payment approximates in value the level of compensation typically provided average workers at the lower end of the wage spectrum for the donation of blood for transfusion purposes. Independently, US$200 is the sum currently paid study subjects who donate bone marrow at UCHSC.

Payments will be stipulated in the individual informed consent forms that subjects sign. Compensation for participation in individual study protocols will be used to defray subject's additional medical expenses. Compensation for giving an interview will be made in cash at the time of the interview. Patients who initially agree to participate in the study will also be informed that in the future they may be eligible for and asked to participate in follow-up sampling. If so, they will be asked to fill out another consent form and compensation will be paid at the time follow-up is requested.

6 PROCEDURAL OVERIVEW AND REGULATORY COMPLIANCE

6.1 Summary of Study Procedures

Blood samples will be collected by venipuncture from in-patients presenting at participating referral hospitals and will be processed for routine CBC, lymphocyte isolation and serum preparation (NHL).

Bone marrow aspirates, bone marrow biopsies, lymph node biopsies and resected tissue received at the clinical laboratories will be subjected to histology, morphologic diagnosis, immunochemical, immunophenotype, cytogenetic, molecular cytogenetics (FISH), and RNA/DNA-based molecular analyses. These studies are complementary to each other and are all essential for diagnoses according to WHO guidelines and as outlined in this proposal. Specimens will be divided into three portions: one for fixation for pathology studies including histology, immunochemistry, and immunophenotype analyses; and one for cell culture for cytogenetics and FISH analyses, one for DNA and RNA isolation.

Initial morphologic diagnoses of lymph node and tumor specimens will be performed by Professor. Zhu and colleagues at FUMC Tumor Hospital or the ICMRC. Morphologic diagnoses will be routinely confirmed and EBV viral status determined using immunophenotype analysis, molecular cytogenetics and RNA/DNA-based molecular analyses on fresh tumor tissue and the same paraffin-embedded tissue sections prepared by FUMC Tumor Hospital.

Morphologic analysis of bone marrow aspirates (BP, AA, MDS, AML) are either performed by Professor Ji of Hua Shan Hospital, RDI and/or JR, and biospy material reviewed RDI and JR. Morphologic diagnoses will be routinely confirmed using immunophenotype analysis, molecular cytogenetics and RNA/DNA-based molecular analyses on bone marrow aspirate slides and paraffin-embedded biopsy sections prepared by FUMC Tumor Hospital. Bone marrow cells obtained from fresh aspiration will be frozen for culture. Individual diagnoses will be reviewed by a panel consisting of the aforementioned individuals and Professor Irons and independently confirmed by Professor John Ryder at UCHSC. A final integrated diagnosis will be made by ICMRC and UCHSC staff on the basis of: Morphologic diagnosis, Immunophenotyping, Cytogenetics, FISH and RNA/DNA-based molecular analysis. This diagnosis will be

communicated to 1) the patient's clinical record and attending physician, and to the Case Control and Disease Progression data bases.

It is likely that some study subjects (%) will be found to possess constitutional (i.e. congenital) chromosome abnormalities during cytogenetic analysis for acquired chromosome changes in bone marrow and lymph nodes. In these cases, the ICMRC laboratory will inform attending physicians of these findings, will offer confirmatory cytogenetic studies on mitogen-stimulated peripheral blood lymphocytes, and will make appropriate recommendations to referral physicians for genetic counseling and genetic clinical evaluation consistent with standards of medical practice in China.

6.2 Regulatory Guidelines and Practice

This study design, review and conduct is being carried out according to Good Epidemiology Practice (GEP) guidelines with specific enhancements as detailed in the Council for International Organizations of Medical Sciences Guidelines (CIOMS 1991,1993) and writings by Brody (1998). Because this is a Multinational Study to be conducted in China, harmonization of clinical laboratory regulatory standards and procedures is required. As much as possible, all procedures performed in the FUMC/UCHSC Joint Clinical and Molecular Laboratory will be carried out in according to the practice standards outlined by the College of American Pathologists (CAP), including QC/QA, with documentation. Different countries have approved different methods and procedures for automated clinical instrumentation and software. Where there are differences, clinical instrumentation and software will be configured to meet Chinese regulatory requirements in preference to US FDA requirements. In compliance with CAP guidelines, immunochemistry and FISH procedures will be performed using automated staining protocols and processing of slides for immunophenotyping, automatic FISH analyses on the paraffin-embedded tissue sections and DNA/RNA isolation from tissue sections. This study will be conducted according to the regulations set forth by the International Collaborative Programme for the use of Chinese Genetic Resources. Study research design, informed consent and clinical protocols outlined in this study as well as protocol amendments affecting study subjects or source data will be subject to review by the COMIRB. The PI will provide the COMIRB with the research proposal, proposed consent forms, questionnaires, data collection forms, subject recruitment materials, scientific and ethical evaluations, as well as progress reports. The PI will also report to the COMIRB any injury, deaths or unexpected adverse experiences encountered by study subjects as a consequence of their participation in this study. In addition, case series of disease prevalence and diagnosis may be published by the ICMRC consistent with Chinese and international guidelines.

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8 ENROLLMENT OF SUBJECTS

8.1 Case Control Study

A Inclusion Criteria

In-patients of either gender 16-75 years of age presenting at any of the participating referral hospitals in Shanghai are candidates for enrollment in this study. The rationale for this age range is based on a relatively young workforce (18-60) and an expected maximum latency of 15 years

for benzene-related AML. Patients will be recruited to the study by Clinical Coordinators on the basis of initial clinical presentation, clinical history and/or previous preliminary blood work.

Patients with severe AA, MDS or AML often present with similar signs and symptoms, which may include: weakness, dizziness, bleeding, petechiae or ecchymoses, less often signs or symptoms of infection, as well as cardiovascular or cerebral symptoms secondary to severe anemia. MDS may present with a chronic anemia of insidious onset and increasing severity. Preliminary examination of peripheral blood may reveal moderate to severe anemia, bi- or tri-lineage cytopenia or dysplasia, pancytopenia and/or a marked increase in circulating abnormal myeloid cells. Individuals presenting with any combination of these signs or symptoms are candidates for enrollment in this study.

Outpatients diagnosed with mild AA or MDS at Central District hospitals will be referred to Hua Shan Hospital for evaluation as Mild Cases. Cases meeting the clinical definition of AA or MDS will be formally recruited to the Disease Progression Study.

B Exclusion Criteria

Patients presenting with any of the following conditions will not be included in this study: subjects with concurrent or previous diagnoses of cancer metastatic to bone marrow, pernicious anemia, paroxysmal nocturnal hemogobinuria, megaloblastic anemia of pregnancy, gastrectomy, hemolytic anemias, anorexia nervosa or congenital anemias (e.g. Fanconi's anemia, Bloom's syndrome, Dyskeratosis Congenita, congenital dyserythropoietic anemias). All hematologic diseases with known heritable or nutritional etiology will be specifically excluded. Additional examples include: congenital red cell aplasia, pernicious anemia, hereditary spherocytosis, hereditary hemoglobinopathies, thalassemias, intrinsic hemolytic anemias, hereditary elliptocytosis, hereditary pyropoikylocytosis, pyruvate kinase deficiency, glucose-6-phosphate dehydrogenase deficiency, megaloblastic anemias resulting from cobalamin or folate deficiencies or iron deficiency anemia.

C Selection of Controls

Controls will be selected for potential cases of AA MDS, MDS/AML and AML that are admitted to referral hospitals. For each case enrolled, one hospital-matched control will be selected. Each control will be matched on hospital, date of diagnosis (nearest to the enrolled case and meeting other criteria), date of birth (+/- 5 yr) and gender. Controls will include any cancer diagnosis except lympho-hematopoietic diseases. Diseases excluded from the control series include: leukemias of any type, pancytopenia, NHL, chronic myeloproliferative disorders, Hodgkin's disease, plasma cell myeloma (multiple myeloma) and paroxysmal nocturnal hemoglobinuria. The over all relationship between the different study sets in this and the accompanying Molecular Epidemiology Study is diagrammed in Figure 8.1 below.

8.2 Benzene Poisoning Case Series

Benzene Poisoning is a compensable occupational disease in China that is defined by the following clinical and exposure criteria: (a) a white count < 4,000/mm^3 or a white count of

between 4,000/mm^3 and 4,500/mm^3 and a platelet count of <80,000/mm^3, (b) work for at least six months in a factory with benzene exposure, and (c) exclusion of other causes of abnormal blood counts. Individuals with previously diagnosed BP that are identified from the SMCDCP database, discovered in the accompanying Molecular Epidemiology Study will be enrolled as cases. If these present to any of the participating tertiary referral hospitals as in-patients, controls will be selected for them. If the BP case is referred by an individual physician outside of Shanghai, it will be enrolled as an individual case. Controls will not be selected for these cases but a full exposure assessment will be performed.

9 WRITTEN INFORMED CONSENT FORM (WICF)

Written informed consent will be obtained from all subjects recruited to this study. Informed consent will be obtained by clinical coordinators from each participating Shanghai referral hospital or by referring physicians for cases outside Shanghai. Clinical coordinators and referring physicians will be familiarized with respect to appropriate conduct/procedures for obtaining informed consent and will undergo training on ethical issues related to informed consent from Professor Xu. Subjects recruited to each disease inclusion category (Control vs NHL vs AA, MDS, AML vs BP) will undergo different procedures or combination of procedures and will receive different compensation as previously discussed. Therefore, individual WICF will be required for each. These will be professionally translated into Mandarin prior to obtaining additional input from Chinese clinical colleagues and review by a Chinese ethicist. Final edited informed consent forms, translated back into English, will be subject to review by the Ethics Panel and the COMIRB.

STANDARDIZED QUESTIONNAIRE FORM

A standardized questionnaire form will be used throughout this study with minor modifications based on presumptive diagnosis (e.g. r/o NHL vs MDS/AML). Final wording of specific questions will be subject to translation and a layered review.

9.1 Personal Information Fields

Patient Identification:
- National Identification Number
- Employer/Health Insurance Number
- Family Name
- First Names

Date of Birth:
Gender:
Address:
Telephone Number:
Date of Admission:
Attending Physician or Clinical Coordinator:
Participating Hospital or Clinic
- Hospital Room Number

Rule Out (Provisional Diagnosis)
Presenting clinical Signs/Symptoms

Date of First Pathologic Diagnosis (If any)

Place of First Pathologic Diagnosis (Hospital/Clinic)

Previous Laboratory Values (Dates):

CBC

- Bone Marrow
- Chemistry

Previous Medical Conditions:
Previous/ongoing Therapy
Employment
- Location
  - SMCDCP Factory #
  - Dates of Employment
  - Job Description
  - Exposures

Previous Employment (1,2,….)
- Location
  - SMCDCP Factory #
  - Dates of Employment
  - Job Description
  - Exposures

Previous known exposure to benzene

Lymphoma Workup:
- Nodal involvement/location:
  - Cervical
  - Waldeyers Ring
  - Supraclavicular
  - Axillary/Pectoral
  - Brachial
  - Hilar
  - Mediastinal
  - Portal
  - Splenic
  - Celiac
  - Abdominal Aortic
  - Iliac/Inguinal/Femoral
  - Other:
- Extranodal involvement
  - Bone Marrow
  - Brain
  - Liver
  - Stomach
  - GI Tract
  - Lung
  - Nasopharyngeal
  - Sinusoidal
  - Other:
- Cytogenetics

Hematology workup:
- WBC
  - Date of analysis
  - Granulocytes Abs
  - Lymphocytes Abs
  - Monocytes Abs
  - Platelets Abs
- RBC
- HCT
- Hgb
- MCV
- MCH
- MCHC
- RDW
- PLT
- MPV
- PDW
- PCT
- RETIC %
- RETIC ABS
- IRF

- Bone marrow
  - Morphology
  - Immunophenotyping
  - Diagnosis

- Cytogenetics

9.2 Significant History/Cofactors/Confounders

Subjects' concurrent and previous medical history will be carefully evaluated for evidence of any of the following: tuberculosis, brucellosis, histoplasmosis, sarcoidosis, parvovirus (B19) infection, HCV or HBV. Subjects will also be queried with respect to possible exposure to any of the following: chloramphenicol, alkylating chemotherapeutic agents, topoisomerase II inhibitors, ionizing radiation, methicillin, penicillamine, clozapine, phenylbutazone, sulfonamides, non-steroidal anti-inflammatory agents, phenatoin, diphenylhydantoin, colchicine, gold salts, allopurinol, quinacrine, carbonic anhydrase inhibitors (e.g. acetazolamide), organophosphates or organic arsenicals. Previous laboratory data will be provided in support of clinical history but will not be reported as study data.

10 IDENTIFICATION OF CLINICAL AND BIOLOGICAL SAMPLES

10.1 Identification Procedures

Identification (ID) codes will be assigned for each new patient subject upon referral. The ICMRC laboratory generates a unique code for each individual, which is stored in each patient's record in the Clinical Database. These ID codes will be cross-referenced with any patient ID codes used by individual hospital or employer's identification numbers. This will allow us to report patient results to participating hospitals using their individual identification numbers while independently ensuring the capability of the laboratory to identify all samples regardless of their origin. The centralized generation of ID codes will guarantee the unique identification of all samples and allow masking of identity when necessary for transfer of data to research databases. Sample masking is essential for confidentiality and to increase the objectivity of the researchers measuring study outcomes. Back-up confirmation will be conducted to assure that there is correspondence between the assigned individual ID codes and those attached to the questionnaire form, consent forms and the biological samples collected, tested and stored. Accession codes will be assigned for individual or groups of tests as appropriate for identification and correlation with individual patients. Labels printed with the patient's ID codes and accession numbers will be included with the sample collection supplies whenever samples are taken. All samples will be labeled with the accession number at all times.

10.2 Access to Clinical and Study Databases

In order to be able to construct study databases, as well as utilize and update clinical and study database records, it is necessary that a small number of personnel have access to both the clinical database (in which subject identifiers are available) and study databases (which do not contain subject identifiers). These will be the only individuals who can link subject identifiers with research data during the course of the study. Such dual access will be strictly limited to the PI, his deputy, and designated clinical laboratory supervisory staff who have an explicit ethical responsibility for maintaining subject confidentiality. The total number of personnel with dual access (which is not expected to exceed six) will include the coordinating clinical hematologist, cytogenetics supervisor, the database manager and a senior technologist assigned the task of validating the accuracy of data entry. Individuals given this authority will be given documented training on acceptable database management procedures and the importance of maintaining subject confidentiality and database integrity.

11 FOLLOW-UP

11.1 AA and MDS

For inpatient cases of AA and MDS, the clinical features of the disease, including bone marrow and cytogenetic examination, will be assessed at 6 month follow-up intervals. The identity of the subject's attending physician will be re-confirmed and results reported to the patient's attending physician to aide in clinical management. The data will also be entered into the study database.

11.2 Benzene Poisoning

For BP clinical features, including bone marrow and cytogenetic examination, results will be reported to the patient's attending physician and be entered into the study database. Newly diagnosed cases of BP will be reported to the subject's physician and the SMCDCP for entry into the BP registry. Newly diagnosed and follow-up cases of BP will be cross-checked with SMCDCP to confirm removal from benzene- exposure environments.

11.3 AML

Cases of AML will not be subject to follow-up, AML being a final diagnosis in the Disease Progression Study. Moreover, no cytogenetic or molecular analyses of clonal abnormalities will be entered into the study database after chemotherapy has been initiated. However, additional follow-up of AML cases will be conducted by the clinical laboratory as requested in support of clinical management of the patient.

12 DIFFERENTIAL DIAGNOSIS AND DISEASE CLASSIFICATION

12.1 AA/MDS

Mild AA clinical features include gradual onset, easy fatigue, mild anemia, infection and/or mild bleeding. If the disease becomes progressively worse over 1-2 year history it is re-designated as severe AA. Peripheral blood parameters are characterized by a gradually decreasing hemoglobin, decreased reticulocyte counts, decreased white count, granulocytes, lymphocytes or platelets.

Severe AA is usually a fulminant illness and may be accompanied by acute symptoms of fatigue, shortness of breath, tinnitis, bleeding, shortness of breath or congestive heart failure. While serious infections are usually rare early in the disease the severity of bleeding is usually related to the degree of thrombocytopenia present. Peripheral blood counts usually reveal markedly reduced numbers of reticulocytes, granulocytes and/or platelets, while monocytes and lymphocytes are variably reduced, and bone marrow cellularity is markedly reduced. Cases of AA can be further characterized with respect to the involvement and severity and of individual cytopenias, i.e. granulocytopenic, lymphocytopenic, thrombocytopenic, or pancytopenic.

Severe MDS can be, and AML is almost always, a fulminant illness that may be accompanied by varying degrees of fatigue, shortness of breath, dizziness, bleeding or infection. Peripheral blood changes can involve anemia and variously either a marked increase or decrease in individual while blood cell counts. It is virtually impossible to distinguish among AA, MDS, CML or AML solely on the basis of clinical presentation, the differential diagnosis requiring morphologic, cytochemical, immunochemical and cytogenetic/molecular analysis of blood and bone marrow (WHO, 2001).

12.2 Mild AA and/or MDS

Peripheral blood parameters in mild AA are characterized by a gradually decreasing hemoglobin, decreased reticulocyte counts, decreased white count, granulocytes, lymphocytes or

platelets. In general, peripheral counts will be higher than for acute aplastic anemia: Reticulocyte count: <2.0%; Hemoglobin: <12 gm/d; WBC: <1.5 x 10^9/L or Granulocytes: <2.0 x 10^9/L or Lymphocytes: <1.0 x 10^9/L or Platelets: <100 x 10^9/L. When available, bone marrow morphology is characterized by bi- or tri- lineage decrease, with at least one site demonstrating hypoplasia. If hyperplasia is present there must be an increased ratio of erythroid precursors and an increase in adipose cells. Mild MDS can present as a pancytopenia or a decrease in one or two lineages which may be accompanied by a macrocytic anemia, large platelets or megaloblastic changes in any lineage. However, there are no uniformly accepted criteria for the distinction between hypocellular MDS and Type I AA, and diagnosis of outpatient subjects with Type I AA/mild MDS will essentially be limited to analysis of peripheral blood counts and smears. These subjects will be followed annually to monitor the status of their condition. Should any individual initially diagnosed with Type I AA/mild MDS progress to a fulminant illness, he or she will be referred to one of the participating teaching hospitals for treatment, differential diagnosis and ascertainment in the case-control study.

12.3 Severe AA

In severe AA peripheral blood counts usually reveal markedly reduced numbers of reticulocytes, granulocytes and/or platelets, while monocytes and lymphocytes are variably reduced. Bone marrow cellularity will be determined from a bone marrow core biopsy, typically cellularity should be between 5-10%. All cell lineages should be involved with no dyspoiesis. If blasts are increased and/or there is dyspoiesis a diagnosis of hypocellular MDS or AML must be considered [108]. AA will be further characterized as to the involvement and severity and of individual cytopenias, i.e. granulocytopenic, lymphocytopenic, thrombocytopenic, or pancytopenic.

12.4 Benzene Poisoning

Benzene poisoning cases will be accepted on the basis of the legally defined criteria established by the Chinese government and will not be independently diagnosed as such by the laboratory.

12.5 Severe MDS

Classification and differential diagnosis of MDS and AML are accomplished according to FAB and proposed WHO criteria. Differential diagnosis of Type II AA and MDS is made on the basis of bone marrow cellularity, morphology and cytogenetic analysis with cases of hypocellular MDS distinguished from Type II AA based on the presence of dyspoietic or megaloblastic changes in bone marrow precursor cells, ringed sideroblasts and/or clonal cytogenetic abnormalities.

A Morphologic analysis of MDS

The microscopic description of the peripheral smear will supplement numerical complete blood count (CBC) data. Morphologic features of red cells (e.g. degree of anisopoikilocytosis, abnormal red cell forms and inclusions) and platelets (e.g. large and giant platelet forms, hypogranularity of platelets) will be described. A 100 white blood cell differential will be performed, and morphologic features of white cells (e.g. blast characteristics including presence

of Auer rods, hypogranularity and hypersegmentation of granulocytes, presence of non-blastic immature granulocytes and monocytes) will be described.

Bone marrow morphology will be evaluated using an H&E-stained and reticulin-stained bone marrow core biopsy, a Wright-Giemsa-stained peripheral smear and aspirate smear, and a Prussian blue-stained aspirate smear. A 400-cell differential will be performed on the aspirate smear. Blast characteristics and any dysplastic features of the three myeloid cell lines will be detailed. Estimation of iron stores and identification and quantification of ringed sideroblasts will be accomplished with Prussian blue-stained aspirate smears.

Descriptions of bone marrow core biopsies will include information on: (1) cellularity; (2) M: E ratio and megakaryocyte content; (3) abnormal localization of immature precursors (ALIP); (4) dysplasia of hematopoietic precursors; (5) stromal abnormalities including increased reticulin [108;109].

B Cytochemical and Immunochemical analysis of MDS

These analytical techniques will be used to confirm morphologic impressions. Air-dried peripheral and bone marrow aspirate smears will be used for cytochemical analysis. Detection of myeloperoxidase in immature/blastic cells indicates that they are neutrophilic, monocytic, or eosinophilic lineage. Detection of non-specific esterase indicates monocytic lineage. Demonstration of inhibition of non-specific esterase activity when alpha naphthyl acetate is used as the substrate increases the specificity of the test for monocytes. Use of alpha naphthyl butyrate also increases the specificity of the test for monocytes. Chloracetate esterase is demonstrable in the non-specific granules of promyelocytes and neutrophils, and in the myeloblasts of some cases of acute leukemia; Auer rods are usually positive [109].

Several markers of myelomonocytic lineage may be used to characterize immature or morphologically obscure cells in myelodysplastic syndromes including myeloperoxidase (air dried smears, flow cytometry or paraffin sections), CD13 (air dried smears or flow cytometry), CD33 (air dried smears or flow cytometry), and CD68 (air dried smears, flow cytometry or paraffin sections). Markers of monocytic lineage include CD14 and CD11c. Glycophorin A will be used as a marker of erythroid lineage; CD42b (air dried smears, flow cytometry or paraffin sections), factor VIII (air dried smears, flow cytometry or paraffin sections) and CD61 will be used as markers megakaryocytic lineage. CD34 (air dried smears, flow cytometry or paraffin sections) expression, if present, confers blast status on cells [109;110].

C Specific diagnostic criteria for MDS subtypes

1. Refractory anemia with and without ringed sideroblasts: The diagnosis refractory anemia with and without ringed sideroblasts will be applied only if abnormalities are restricted to the erythroid line [111]. Dyserythropoiesis will be determined by examining erythroid precursors in a bone marrow aspirate smear. The myeloid blast count should be less than 5% of all nucleated cells (ANC) counted on a bone marrow aspirate smear. The diagnosis refractory anemia with ringed sideroblasts will be applied if greater than 15% of all nucleated cells in a Prussian blue-stained aspirate smear are ringed sideroblasts [108;112;113].

2. Refractory cytopenia with multilineage dysplasia: This diagnosis will be applied to cases where less than 5% of all nucleated cells in the bone marrow are blasts, but there are dysplastic features in two or more cell lines (erythroid, granulocytic, megakaryocytic). Ringed sideroblasts are variably present [112].

3. Refractory anemia with excess blasts: This diagnosis will be applied to cases where there are greater than 5% but less than 20% blasts in the bone marrow. There is generally dysplasia of erythroid, granulocytic, and megakaryocytic cell lines [108;112].

4. 5q- syndrome: This syndrome is associated with macrocytosis and thrombocytosis in the peripheral blood, and erythroblastopenia and prominent hypolobulated megakaryocytes in the bone marrow. Demographically this tends to be a disease of elderly women. 5q- is the sole karyotypic abnormality. Most patients have less than 5% blasts in the bone marrow [108;108;109;112;113].

5. Chronic Myelomonocytic Leukemia (CMML): This syndrome is classified as a FAB subtype of MDS but as Myelodysplastic/ Myeloproliferative Syndrome under the WHO Classification. CMML has characteristics of refractory anemia (myelodysplastic syndrome) with excess blasts, although dyserythropoiesis may not be prominent. The defining feature of this disorder is a peripheral monocytosis of $>10^9$/L, although monocytic hyperplasia may not be noted in the bone marrow [108;112].

12.6 AML

General considerations (morphology, cytochemical and immunochemical analysis, and molecular/genetic analysis) for AML are the same as for MDS. Using morphologic, cytochemical and immunochemical data, a given case of AML will be classified according to the criteria of the French-American-British (FAB) Study Group (FAB M0, M1, M2, M3, M4, M5, M6, M7) [108]. Additional cytochemical/immunochemical, historical (e.g. previous chemotherapy, previous myelodysplastic syndrome), and molecular/genetic data will be used to further classify a particular AML according to proposed WHO criteria [112]. A critical difference between WHO and FAB criteria involves the definition of acute leukemia: 30% blasts is necessary for a diagnosis of acute leukemia according to the FAB system while only 20% blasts is necessary for a diagnosis of acute leukemia according to the WHO system [112]. Current practice in China adopts a 20% cutoff for diagnosis of AML.

A AMLs with recurrent Cytogenetic Translocations:

1. AML with t(8;21)(q22;q22) This translocation is usually found in AMLs meeting criteria for FAB M2 [114;115]. If this translocation is found the case will be considered to be AML even if there is a low blast count (i.e. < 20%) [112].

2. AML with t(15;17)(q22;q11-12) and variants (t(11;17)(q23;q11), t(5;17)(q31;q11), t(11;17)(q13;q11. Of chromosomal abnormalities involving the retinoic acid receptor (RAR) gene on chromosome 17, t(15;17)(q22;q11-12) is by far the most common [114;115].

Chromosomal abnormalities involving the RAR gene are exclusively associated with acute promyelocytic leukemia (FAB M3), and are nearly always found in this disorder [114;115]. If this translocation involving the RAR gene are found the case will be considered to be AML even if there is a low blasts count (i.e. < 20%)[112].

3. AML with inv(16)(p113;q22). This translocation is associated with abnormal bone marrow eosinophils. It is found in AML meeting the criteria for FAB M4, and occasionally FAB M2 [114;115]. If this translocation is found the case will be considered to be AML even if there is a low blast count (i.e. < 20%) [112].

4. AML with 11q23 (MLL) abnormalities. Translocations involving 11q23 are the most common in human acute leukemia and involve a number of different partner genes [114;115]. AMLs with 11q23 abnormalities do not consistently meet criteria for a particular FAB subtype, although they are frequently associated with M4 and M5. In fact 11q23 abnormalities may also be found in acute lymphoblastic leukemia and lymphoma [114]. 11q23 abnormalities are the most common translocations in secondary leukemias associated with topoisomerase II inhibitors [112].

B AML's with Multilineage Dysplasia

Multilineage dysplasia is defined as dysplastic features in two or more cell lines [112]. This category will be further subdivided into cases with an antecedent myelodysplastic syndrome and cases without an antecedent myelodysplastic syndrome [112].

1. Therapy-related AMLs

This category will be further subdivided into AMLs secondary to treatment with alkylating agents, AMLs secondary topoisomerase II inhibitors, and AMLs secondary to other chemotherapeutic agents [112].

AML not otherwise classified

Entities in this category do not fall into any of the previously described categories:

a. AML minimally differentiated FAB M0 criteria (except for blast count) will apply: ≥20% blasts; <3% positivity for myeloperoxidase as assessed by conventional cytochemistry; myeloid antigen expression by immunocytochemistry [108].

b. AML without maturation FAB M1 criteria (except for blast count) will apply: ≥20% blasts; ≥3% positivity for myeloperoxidase in blasts as assessed by conventional cytochemistry; <10% of cells exhibiting maturation beyond the blast stage [108].

c. AML with maturation FAB M2 criteria (except for blast count) will apply: ≥20% blasts; ≥3% positivity for myeloperoxidase in blasts as assessed by conventional cytochemistry; >10% of granulocytic cells exhibiting maturation beyond the blast stage; <20% of cells positive for non-specific esterase [108].

d. Acute myelomonocytic leukemia FAB M4 criteria (except for blast count) will apply: ≥20% of cells are myeloblasts, monoblasts and/or promonocytes; >20% positivity for myeloperoxidase in cells as assessed by conventional cytochemistry; >20% of cells positive for non-specific esterase [108].

e. Acute monocytic leukemia FAB M5 criteria (except for blast count) will apply: ≥20% of cells are myeloblasts, monoblasts and/or promonocytes; <20% positivity for myeloperoxidase in cells as assessed by conventional cytochemistry; >80% of cells positive for non-specific esterase [108].

f. Acute erythroid leukemia FAB M6 criteria (except for blasts count) will apply: ≥20% of non-erythroid cells are myeloblasts; >50% of all nucleated cells are erythroid precursors [108].

g. Acute megakaryocytic leukemia FAB M7 criteria (except for blast count) will apply: ≥30% of cells are myeloblasts and/or megakaryoblasts; >30% of cells are megakaryocytic as determined by immunochemistry [108].

12.7 Diagnostic Criteria for Exclusion of Myeloid Diseases

A General Considerations

Diagnostic criteria are provided for hematologic conditions that are not under study but that will invariably be encountered, either during triage for case ascertainment or in the course of clinical activities, and will need to be distinguished in the differential diagnosis of diseases in this study.

B Chronic Myelogenous Leukemia (CML).

This entity is a clonal myeloproliferative disorder that is generally manifested by a leukemic granulocytosis and splenomegally. During the chronic phase peripheral leukocyte counts may vary between 10^9-10^{12} cells/L. Relatively mature segmented granulocytes and bands predominate followed in decreasing order by metamyelocytes, myelocytes, promyelocytes and myeloblasts. These are virtually always accompanied by an absolute increase in basophils and a high peroxidase content that forms a broad band versus cell size on automated instrumentation. These abnormalities are accompanied by myelodysplastic changes in all cell lineages. Cytochemical analysis usually shows a weak or negative leukocyte alkaline phosphatase (LAP) reaction and positive myeloperoxidase and alpha-naphthol AS-D chloroacetate esterase reactions. The *sine-quo-non* of CML is the presence of the t(9;22) translocation, i.e. the Philadelphia Chromosome, and/or demonstration of the BCR-ABL fusion product which forms a 210 kd translation product. The presence of this clonal abnormality is sufficient to exclude a diagnosis of MDS, and in the acute myeloid blast phase, CML may be distinguished from AML as well based on the presence of t(9;22). The differentiation between CML in lymphoid blast phase and pH-positive ALL can sometimes be made on the basis of a smaller fusion product, P190, occurring in about half of ALL cases [116;117].

C Precursor B-lymphoblastic lymphoma/leukemia

The term acute lymphoblastic leukemia (ALL) will be retained for malignancies of pre-B-cells and pre-pre-B-cells that primarily involve the blood and bone marrow [111;112]. Morphologic considerations: The FAB L1 and L2 morphologic categories have proven not to be predictive of immunophenotype, genetic abnormalities, or clinical behavior [118]. Thus L1 or L2 morphology will not be designated when a pre-B or pre-pre-B-lymphoblastic malignancy is diagnosed. A malignancy of pre-B-cells or pre-pre-B-cells may have a lymphomatous or leukemic presentation and still be considered the same disease.

1. Immunochemistry of ALL

The pre-pre-B-cell acute lymphoblastic malignancy variably express B-cell markers (CD19 (air dried smears or flow cytometry (F)), CD20 (air dried smears, flow cytometry or paraffin sections (FP)), CD79a (FP), TdT (FP), and may or may not express CD10 (FP); "common" pre-pre-B-cell lymphoblastic leukemia expresses CD10. Expression of cytoplasmic IgM is indicative of a pre-B-cell acute lymphoblastic malignancy [112].

12.8 NHL

A General Considerations

Cases will be classified according to the WHO classification supplemented with anatomical and clinical information that may be important in diagnosis and etiology as well as markers that might be useful in ascertaining potential cofactors or confounders (e.g. viral genes). Tumors will be classified according to defining histology, immunophenotype and clonal cytogenetic and molecular abnormalities. In addition, anatomical site will be noted (i.e. nodal, extranodal, Waldeyers' ring sinonasal/nasopharyngeal, mediastinal, gastric, intestinal, epidural, cutaneous, body cavity or CNS). Histologic sections will be analyzed for expression of EBER - EBV gene expression. In addition all tumors will be analyzed for HCV and p53; gastric lymphomas will be analyzed for *Helicobacter pylori*; cutaneous T cell lymphomas will be analyzed for HTLV-I. Patients' sera will be screened for HBV and HCV.

B B-Cell Neoplasms

B.1 Precursor B-lymphoblastic lymphoma/leukemia

Morphologic considerations: The FAB L1 and L2 morphologic categories have proven not to be predictive of immunophenotype, genetic abnormalities, or clinical behavior [118]. Thus L1 or L2 morphology will not be designated when a pre-B or pre-pre-B-lymphoblastic malignancy is diagnosed. A malignancy of pre-B-cells or pre-pre-B-cells may have a lymphomatous or leukemic presentation and still be considered the same disease. However the term acute lymphoblastic leukemia (ALL) will be retained for malignancies of pre-B-cells and pre-pre-B-cells that primarily involve the blood and bone marrow [111;112]. The L3 morphologic category generally corresponds to Burkitt's lymphoma/Burkitt-cell leukemia (see below), and should correspond with immunophenotypic and genetic findings [118].

Immunophenotype: The pre-pre-B-cell acute lymphoblastic malignancy variably express B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)), TdT (FP), and may or may not express CD10 (FP); "common" pre-pre-B-cell lymphoblastic leukemia expresses CD10. Expression of cytoplasmic IgM is indicative of pre-B-cell acute lymphoblastic malignancy [110].

B.2 Burkitt's lymphoma/Burkitt-cell leukemia

Morphologic considerations: As indicated, the FAB L3 lymphoblast is the Burkitt cell. As with pre-B cell and pre-pre-B-cell malignancies, malignancies of Burkitt cells are biologically the same disease whether they present as lymphoma or leukemia [111;112]. Malignancies of cells that do not conform well with the L3 morphology ("Burkitt-like", "non-Burkitt's") will be considered variants of Burkitt's lymphoma if c-*myc* rearrangements can be detected and the proliferation of cells is near 100%; in the absence of cytogenetic data, the latter criterion will suffice for a diagnosis of variant Burkitt's lymphoma [111;112].

Immunophenotype: Malignant cells variably express B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) and CD10 (FP). Cells are negative for TdT [118]. The Ki-67 (FP) fraction should close to 100% [111;112].

Molecular analysis: Burkitt's lymphomas exhibit 8q24 cytogenetic abnormalities which are confirmed by FISH analysis for rearrangement of c-MYC.

B.3 B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma

Morphologic considerations: B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma will be considered the same disease entity with different clinical manifestations [111;112]. Although its prognostic significance is still not clear, the presence of plasmacytoid differentiation does not indicate a different disease [112]. Lymph nodes and bone marrow involved by B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma have a fairly distinct appearance: a diffuse infiltrate of small "mature" lymphocytes with interspersed pseudofollicular proliferation centers [118]. However, to avoid any confusion with other neoplasms of small mature lymphocytes (e.g. mantle-cell lymphoma, marginal zone B-cell lymphoma), immunophenotyping will be carried out.

Immunophenotype: Malignant cells faintly express surface IgM (F) or less frequently IgD (F). There is variable expression B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)). Co-expression of CD5 (FP), CD43 (FP), and CD23 (FP) along with the variably expressed B-cell markers and faintly expressed surface immunoglobulin is characteristic of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma [118].

B.4 B-cell prolymphocytic leukemia

See Precursor B Cell Lymphoma/Leukemia

B.5 Lymphoplasmacytic lymphoma

Morphologic considerations: This entity is comprised of a diffuse infiltrate of cells that display a spectrum of features from small "mature" lymphocytes to plasmacytoid lymphocytes to plasma cells. Dutcher bodies may or may not be found. This neoplasm is not to be confused with other B-cell tumors that may display plasmacytoid differentiation and should lack distinctive features of these other B-cell tumors (e.g. pseudofollicular proliferation centers seen in B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma). Most cases of Waldenstrom's macroglobulinemia are a consequence of this disorder [118].

Immunophenotype: In keeping with its association with Waldenstrom's macroglobulinemia, this neoplasm is usually positive for surface IgM (F) and cytoplasmic IgM (FP). B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) are positive. In contrast to B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma and mantle-cell lymphoma, CD5 (FP) is negative. CD43 (FP) is variably positive and CD10 (FP) is negative [118].

B.6 Mantle-cell lymphoma

Morphologic considerations: The neoplastic cell of mantle-cell lymphoma has a variable cytologic presentation. Usually the cells are slightly larger than a small "mature" lymphocyte, have somewhat irregular or cleaved nuclei, inconspicuous nucleoli and scant cytoplasm. However cells may have rounder nuclei that resemble small "mature" lymphocytes, or may even have a lymphoblastic appearance. The neoplastic infiltrate is usually diffuse of "vaguely nodular"; in rare instances a follicular pattern may be seen. Residual benign germinal centers surrounded by broad expanses of neoplastic cells are known as naked germinal centers. The presence of epithelioid histiocytes may impart a "starry sky" appearance to the neoplasm at low magnification [118]. The prognostic/biological/therapeutic significance of the pattern of the neoplastic infiltrate (diffuse vs. vaguely nodular) and the cytologic appearance of the neoplastic cell has not been determined. Thus pattern and cytologic features of a mantle-cell lymphoma will be described but not used for grading or subclassification purposes [112].

Immunophenotype: The neoplastic cells of mantle-cell lymphoma are positive for surface immunoglobulin, usually IgD (F); lambda light chain (F) expression predominates over kappa light chain (F) expression. Surface immunoglobulin expression is brighter in mantle-cell lymphoma than it is in B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma. B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) are positive. Similarly to B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, mantle-cell lymphoma cells express CD5 (FP); in contrast to B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma they do not express CD23 (FP). CD43 (FP) is positive. CD10 (FP) is variably positive. Detection of cyclin D1 (FP) expression results from the t(11;14) chromosomal translocation that is found in the majority of cases of mantle-cell lymphoma [118].

Molecular analysis: Cells exhibit t(11;14)(q13;q32) which is confirmed by FISH for BCL 1/IgH and PCR.

B.7 Extranodal marginal zone B-cell lymphoma of MALT type

Morphologic considerations: Extranodal marginal zone B-cell lymphoma of MALT type usually involves glandular epithelial tissue, although the tumor may also arise in skin and soft tissue. The most common site of involvement is the stomach. This tumor is associated with autoimmune disease (e.g. Sjogren's syndrome, Hashimoto's thyroiditis) and in gastric lesions, *Helicobacter pylori*. Thus an antecedent or concurrent history of an autoimmune disease or identification of *H. pylori* will be reported when this neoplasm is diagnosed. The neoplastic infiltrate is heterogeneous consisting of variable numbers of marginal zone cells (so-called "centrocyte-like" cells), monocytoid B-cells, plasma cells, and small round lymphocytes. The neoplastic infiltrate is located in marginal zones or interfollicular areas between reactive follicles, which are usually present. Follicles may be invaded and overrun by neoplastic cells ("follicular colonization"). The epithelium of epithelial tissues containing this tumor is usually focally (at least) infiltrated by malignant marginal zone cells to form lymphoepithelial lesions [118]. The significance of the presence of increasing numbers of centroblastic or immunoblastic cells within an extranodal marginal zone B-cell lymphoma of MALT type is not clear, although prominence of intermixed centroblastic and/or centrocytic cells should be noted. If diffuse sheets of large neoplastic cells are identified the term "high-grade malt lymphoma" will not be used. Rather a diagnosis of diffuse large B-cell lymphoma with areas of marginal zone/MALT lymphoma will be made [111;112].

Immunophenotype: Neoplastic cells express surface immunoglobulin (F) (IgM, IgG, or IgA) and variably express cytoplasmic immunoglobulin (FP). B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) are positive. CD43 (FP) and Bcl-2 (FP) are variably expressed. CD5 (FP), CD10 (FP), and CD23 (FP) are negative.

Molecular analysis: Cells may exhibit t(11;14)(q32;q21) or trisomy 3,7 or 12 which can be confirmed by FISH.

B.8 Nodal marginal zone B-cell lymphoma

Morphologic considerations: This tumor will be distinguished from extranodal marginal zone B-cell lymphoma of MALT type with lymph node involvement – i.e. the diagnosis of nodal marginal zone B-cell lymphoma will be made only if the neoplasm is found exclusively in lymph nodes [112]. Neoplastic marginal zone cells are typically found in perisinusoidal, parafollicular or marginal zone areas [118]. See extranodal marginal zone B-cell lymphoma of MALT type for additional details.

Immunophenotype: See extranodal marginal zone B-cell lymphoma of MALT type.

B.9 Splenic marginal zone B-cell lymphoma

Morphologic considerations: This tumor will be distinguished from extranodal marginal zone B-cell lymphoma of MALT and extranodal marginal zone B-cell lymphoma of MALT type (2). Splenic mantle and marginal zones of the white pulp as well as the red pulp may be involved; "villous lymphocytes" may be present [118]. See extranodal marginal zone B-cell lymphoma of MALT type for additional details.

Immunophenotype: See extranodal marginal zone B-cell lymphoma of MALT type.

B.10 Plasma cell myeloma/plasmacytoma

Morphologic considerations: These neoplasms are composed predominantly of plasma cells without the prominent intermixture of lymphocytes and lymphoplasmacytoid cells such as may be seen in other lymphoid tumors (e.g. lymphoplasmacytic lymphoma). Plasma cells may have features of atypia and immaturity; plasma cells with blastic features ("plasmablasts") may be present. Plasma cell neoplasms usually present as disseminated skeletal tumors (multiple myeloma), but may also present as solitary lesions in bone or other tissues. Reports will indicate if a plasma cell tumor is solitary or multiple if known [118].

Immunophenotype: Tumor cells are positive for cytoplasmic immunoglobulin (FP) (IgG, IgA, rarely IgD, IgE). Tumor cells may also express cytoplasmic kappa or lambda light chains only (FP), or rarely heavy chains only (FP). Tumor cells are also positive for CD38 (F), and are variably positive for CD79a (FP), CD45 (FP), HLA-DR (FP), EMA (FP), CD43 (FP), and CD56 (FP). Tumor cells are negative for the pan-B cell antigens CD19 (F), CD20 (FP), and CD22 (FP) [118].

B.11 Hairy cell leukemia

Morphologic considerations: The neoplastic cell and bone marrow and red pulp splenic infiltrate of hairy cell leukemia are fairly distinctive, although there is a possibility of confusion with neoplasms that contain a high concentration of monocytoid B-cells. Therefore all suspected cases of hairy cell leukemia will be immunophenotyped [118].

Immunophenotype: Hairy cells are positive for surface immunoglobulin (F). B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) are positive. CD11c (F), CD103 (FP), CD25 (FP), and FMC7 (F) are also positive. CD5 (FP), CD10 (FP), and CD23 (FP) are negative [118]. Detection of tartrate-resistant acid phosphatase in hairy cells may be detected using conventional cytochemical methods or by immunochemistry (FP) [110]. DBA44 (FP) is also used for the diagnosis of hairy cell leukemia [110].

B.12 Follicular lymphoma

Morphologic considerations: Follicular lymphomas will be graded by the relative content of large and small cells in neoplastic follicles: grade 1 predominantly small cleaved cell; grade 2 mixed small cleaved and large cell; grade 3 predominantly large cell [111;112;118]. The Berard criteria will be used to discriminate between grades 1, 2 and 3: grade 1, 0 to 5 large cells ("centroblasts") per high power field (hpf); grade 2, 6 to 15 centroblasts/hpf; grade 3, greater than 15 centroblasts/hpf [111;112]. The tumor will be described as predominantly follicular if greater than 75% of the area examined has a follicular pattern, follicular and diffuse if 25% to 75% of the area has a follicular pattern, and predominantly diffuse if less than 25% of the area has a follicular pattern [111;112].

Immunophenotype: Tumor cells are variably positive for surface immunoglobulin (F), CD10 (FP), CD23 (FP), and B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) [118]. Bcl-2 expression (FP) is detected in 100% of grade 1 follicular lymphoma, 86% of grade 2 follicular lymphoma, and 76% of grade 3 follicular lymphoma [110]. Tumor cells are negative for CD5 (FP), CD43 (FP), and CD11c (F) [118].

Molecular analysis: Cells usually exhibit t(14;18)(q32;q21) which can be confirmed by FISH for BCL 2/IgH and PCR.

B.13 Diffuse large B-cell lymphoma

Morphologic considerations: Although diffuse large B-cell lymphoma (DLBCL) will not be classified according to the cytomorphology of the neoplastic cells, cytomorphology will be described. Cytomorphologic categories include centroblastic, immunoblastic, anaplastic large cell, and plasmablastic. Additionally features such as prominence of T-cells and histiocytes (T-cell rich/histiocyte rich) will be described. The primary site of any DLBCL will be specified (e.g. nodal, mediastinal, intravascular, primary effusion) [111;112].

Immunophenotype: Tumor cells variably express surface immunoglobulin (F) and/or cytoplasmic immunoglobulin (FP). B-cell markers (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)) are positive. There is variable expression of CD45 (FP), CD5 (FP), CD10 (FP), CD30 (FP), and Bcl-2 (FP) [118].

Molecular analysis: Cells can exhibit t(14;18)(q32;q21); t(3;14) or t(8;14) which can be confirmed by FISH for BCL 2, BCL6 or c-MYC and PC.

C T-Cell Neoplasms

C.1 Precursor T-lymphoblastic lymphoma/leukemia

Morphologic considerations: As with precursor B-lymphoblastic lymphoma/leukemia, L1 and L2 terminology will not be used (although blasts will be carefully described). Biologically T-lymphoblastic leukemia and lymphoma will be considered the same disease with different clinical presentations.

Immunophenotype: Early T lymphoblastic lesions are positive for CD7 (F) and CD34 (FP), and variably positive for TdT (FP). Immature thymocyte lymphoblastic lesions are positive for CD7 (FP), TdT (FP), CD5 (FP), CD38 (F), CD2 (F), and cytoplasmic CD3 (FP), and variably positive for CD34 (FP). Common thymocyte lymphoblastic lesions are positive for CD7 (FP), TdT (FP), CD5 (FP), CD38 (F), CD2 (F), CD1 (FP), CD3 (FP), CD4 (FP), and CD8 (FP). Mature thymocyte lymphoblastic lesions are positive for CD7 (FP), CD5 (FP), CD38 (F), CD2 (F), CD3 (FP), and CD4 (FP) or CD8 (FP), and variably positive for TdT (FP) [108].

Molecular analysis: A variety of structural deletions are encountered, including: 14q11-; 7q34-; 7p15-; 9q21-, p16.

C.2 Peripheral T-cell lymphoma, not otherwise characterized

Morphologic considerations: Peripheral T-cell neoplasms that are not part of one of the syndromes described below will be diagnosed as peripheral T-cell lymphoma, not otherwise characterized. Thus these tumors would not be subcategorized according to cytomorphology or pattern. However cytomorphology will be described carefully (e.g. small vs large cells, mixed small and large cell, immunoblastic) as will pattern and any intermixed benign cell population (e.g. lymphoepithelioid – Lennert's – cell lymphoma) [111;112;118].

Immunophenotype: The pan-T-cell antigens CD2 (F), CD3 (FP), CD5 (FP), and CD7 (F) are variably expressed by peripheral T-cell tumors. 81% of peripheral T-cell tumors will express 2 or more pan-T-cell antigens; CD2 and CD3 are most frequently expressed. CD4 (FP) is expressed by 47 to 58% of peripheral T-cell neoplasms, while CD8 is expressed by 7% to 18%. Other antigens that may be expressed include CD45 (FP) and CD45RO (FP) [112;118].

C.3 Mycosis fungoides/Sezary syndrome

Morphologic considerations: Analysis of cases of mycosis fungoides/Sezary syndrome will include evaluation of cutaneous lesions, the extent of lymph node (and other organs) and peripheral blood involvement. Ideally analysis of peripheral blood involvement will accomplished using flow cytometry; at the very least the number of atypical, Sezary-like cells as a percentage of total white blood cell count and absolute lymphocyte count will be determined. Lymph node involvement will be assessed using NCI-Navy Criteria [113].

Immunophenotype: Tumor cells express the T-cell antigens CD2 (F), CD3 (FP), and CD5 (FP); CD7 (F) is negative in around one third of cases. Tumor cells are generally CD4 (FP) positive; CD8 (FP) and CD25 (FP) are rarely positive [118].

C.4 Angioimmunoblastic T-cell lymphoma

Morphologic considerations: Morphologic features of this tumor include lymph node effacement, proliferation of high endothelial venules, aggregates if follicular dendritic cells, "burnt-out" germinal centers, and an infiltrate of immunoblasts and atypical lymphocytes. In keeping with current thinking based on T-cell receptor gene re-arrangement studies, this lesion will be considered a lymphoma rather than an abnormal immune response.

Immunophenotype: Tumor cells express pan-T-cell antigens CD2 (F), CD3 (FP), CD5 (FP), and CD7 (F). CD4 (FP) is usually positive.

C.5 Anaplastic large-cell lymphoma, T/null cell, systemic

Morphologic considerations: This tumor is generally described as being composed of large, pleomorphic often bizarrely shaped cells. However, two variants are recognized and will be noted if encountered: a lymphohistiocytic variant and a small cell variant. A diagnosis of anaplastic large-cell lymphoma, T/null cell, systemic will be made regardless of whether a t(2;5) translocation is identified and/or the cell expresses the ALK protein [111;112].

Immunophenotype: Tumor cells are CD30 (FP) positive and variably positive for EMA (FP). T-cell tumors variably express T-cell-associated markers (CD3 (FP), CD43 (FP), CD45RO (FP)); null-cell tumors do not [118].

C.6 Anaplastic large-cell lymphoma, T/null cell, primary cutaneous type

Morphologic considerations: The cutaneous form of anaplastic large-cell lymphoma has a more indolent clinical course than the systemic form, usually the t(2;5) translocation and usually does not express EMA. Thus it is presently considered to be a distinct disease and is placed in its own category rather than being subsumed under the systemic category [111;112;118].

Immunophenotype: See anaplastic large-cell lymphoma, T/null cell, systemic. EMA is negative.

C.7 T-cell prolymphocytic leukemia

Morphologic considerations: The tumor cells have somewhat irregular nuclei with clumped chromatin, prominent nucleoli, and fairly abundant cytoplasm. Bone marrow involvement tends to be diffuse. Tumor cells form a diffuse infiltrate in paracortical regions of lymph nodes; pseudofollicular proliferation centers such as are seen in B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma are absent. Splenic red pulp and hepatic sinusoids may also be infiltrated by neoplastic cells [118].

Immunophenotype: Tumor cells express pan-T-cell antigens CD2 (F), CD3 (FP), CD5 (FP), and CD7 (F). CD4 (FP) is usually positive, although cells may also be positive for CD4 (FP) and CD8 (FP). CD25 (FP) is negative.

C.8 T-cell granular lymphocytic leukemia

Morphologic considerations: The neoplastic cells of this disorder are typically called large granular lymphocytes. These cells have a round to oval nucleus with moderately condensed chromatin. They have abundant pale blue cytoplasm containing small, scattered azurophilic granules. Bone marrow involvement is often sparse and difficult to identify. The splenic red pulp and hepatic sinuses may be involved [118].

Immunophenotype: The phenotype of neoplastic cells is referred to as NK-like T-cell since both NK and T-cell-associated antigens are expressed. T-cell-associated antigens include CD2 (F), CD3 (FP), and CD8 (FP). NK-associated antigens include CD16 (F), and CD57 (FP); CD56 (FP) is variably expressed [118].

C.9 Aggressive NK-cell leukemia

Morphologic considerations: See T-cell granular lymphocytic leukemia. Tumor cells in this category may look like large granular lymphocytes or they may assume a more lymphoblast-like morphology [118].

Immunophenotype: Tumor cells have a purer NK cell phenotype in that markers of mature T-cells are absent. Neoplastic cells are positive for cytoplasmic CD3 (FP), CD56 (FP), and CD16 (F); cells are variably positive for CD57 (FP).

C.10 Adult T-cell lymphoma/leukemia (HTLV1+)

Morphologic considerations: By definition this neoplasm is caused by the HTLV1. Thus there will be serologic and/or molecular evidence of HTLV1 infection in all cases of lymphoma/leukemia assigned this diagnosis. Neoplastic cells in the peripheral blood and in diffuse infiltrates in lymph nodes, bone marrow, and other organs range in size from small to large and usually appear markedly atypical (e.g. "clover leaf" and "fleur de lis" cells) [118].

Immunophenotype: The T-cell-associated antigens CD2 (F), CD3 (FP), and CD5 (FP) are usually expressed; CD7 (F) expression is usually absent. Tumor cells are CD4 (FP) and CD25 (FP) positive, and rarely CD8 (FP) positive [118].

C.11 Extranodal NK/T-cell lymphoma, nasal type

Morphologic considerations: The tumor cell infiltrate in this disorder is characteristically angiocentric and angioinvasive. Small "mature" lymphocytes, variably sized atypical lymphocytes, immunoblasts, eosinophils and histiocytes are within the infiltrate. Invasion of vascular walls may be accompanied by occlusion of vascular lumens by atypical lymphoid cells resulting in ischemic necrosis of tumor and normal tissue [118].

Immunophenotype: Tumor cells are positive for CD2 (F), CD56 (FP), cytoplasmic CD3 (FP), CD45RO (FP), CD43 (FP), and EBER (EBV by *in situ* hybridization. CD7 (FP), CD4 (FP), and CD8 (FP) are occasionally positive [119].

C.12 Enteropathy-type-T-cell lymphoma

Morphologic considerations: This tumor usually occurs in the setting of gluten-sensitive enteropathy. However it may also occasionally occur in the absence of any evidence of enteropathy. Thus the diagnosis of enteropathy-type-T-cell lymphoma will be made in the absence of enteropathy as long as histologic and immunophenotypic features are consistent. Tumors typically have variably sized anaplastic cells with a high content of mucosal intraepithelial T-cells [118].

Immunophenotype: Tumor cells are positive for CD3 (FP), CD7 (F), and CD103 (FP). Cells are variably positive for CD8 (FP).

C.13 Hepatosplenic gamma-delta T-cell lymphoma

Morphologic considerations: This tumor, which is commonly seen in young males, affects the spleen and liver; hepatosplenomegly is almost invariably present at presentation. The bone marrow may also be involved. Moderately sized tumor cells with round to slightly irregular nuclei infiltrate demonstrate a sinusoidal infiltration pattern in the spleen and liver. Portal tracts and white pulp are partially spared [113].

Immunophenotype: Tumor cells are positive for CD3 (FP), and are usually positive for CD2 (F) and CD7 (F); CD5 (FP), CD4 (FP), and CD8 (FP) are generally negative. Since this is a neoplasm of γ/δ T-cells TCRδ (F) is positive and βF1 (FP) is negative [110;113].

C.14 Subcutaneous panniculitis-like T-cell lymphoma

Morphologic considerations: Patients present with subcutaneous nodules that may resemble chronic panniculitis or erythema nodosum. Tumor cells are variably sized with hyperchromatic, somewhat irregularly shaped nuclei. The tumor infiltrate is largely confined to the subcutaneous tissue with little extension into the dermis. Cells tend to infiltrate between adipocytes; karyorrhexis and fat necrosis are common. Extra-cutaneous spread is uncommon [113].

Immunophenotype: The pan-T-cell antigens CD2 (F), CD3 (FP), CD5 (FP), and CD7 (F) are variably expressed by tumor cells. Both γ/δ and α/β T-cell tumors have been described, so neoplastic cells are variably positive for TCRδ (F) and βF1 (FP) [113].

12.9 Diagnostic Criteria for Exclusion of Hodgkin's Lymphoma (Disease)

Hodgkin's disease (HD) is specifically excluded from the case control study but because of the similarities in presentation and pathology cases of HD will necessarily have to be diagnosed. Therefore, criteria for diagnosis of HD are also included.

A Nodular Lymphocyte-Predominant Hodgkin's Lymphoma

Morphologic considerations: This entity has been separated from "classical" Hodgkin's lymphoma (see below) because of unique morphologic and phenotypic characteristics. Morphologic features include a nodular growth pattern that may or may not have diffuse areas. Progressively transformation of germinal centers may precede the development of this tumor, or may occur concurrently in the same or other lymph nodes. The characteristic cell of this tumor is the "lymphocytic and/or histiocytic" (L&H) cell. The L&H cell has polylobated, vesicular nucleus with small nucleoli and is sometimes referred to as a "popcorn" cell. Diagnostic Reed-Sternberg cells are usually not found. The non-neoplastic milieu is composed predominantly of small lymphocytes; there may also be clusters of epithelioid histiocytes. Plasma cells, eosinophils and neutrophils are not prominent [118].

Immunophenotype: L&H cells are positive for B-cell associated antigens (CD19 (F), CD20 (FP), CD22 (FP), CD79a (FP)). EMA (FP) and CD30 (FP) are variably positive. CD15 (FP) and surface (F) and cytoplasmic (FP) immunoglobulin is negative. Most of the small lymphocytes in the milieu of the nodules are B-cells. However T-cells are plentiful and CD57 (FP) positive T-cells tend to surround L&H cells [110;118].

B Classical Hodgkin's Lymphoma

B.1 Nodular sclerosing Hodgkin's lymphoma (grades 1 and 2)

Morphologic considerations: Although nodular areas separated by birefringent fibrous bands are characteristic of this tumor, diffuse areas may be present. The Reed-Sternberg variant that is characteristic of this neoplasm is the lacunar cell. Diagnostic Reed-Sternberg cells are also usually found. The clinical usefulness of assessing the number of Reed-Sternberg cells and variants is unclear. In order to facilitate further studies on this matter, these tumors will be graded: grade 1, few Reed-Sternberg cells and variants; grade 2, many Reed-Sternberg cells and variants. The non-neoplastic milieu is composed of small lymphocytes, histiocytes, plasma cells, eosinophils, and neutrophils. Areas of necrosis are not infrequent [111;112;118].

Immunophenotype: Reed-Sternberg cells and variants are typically positive for CD30 (FP) and CD15 (FP); CD15 may occasionally be negative. In 20% of cases a B-cell-associated antigen, and in 10% of cases a T-cell-associated antigen. EMA (FP) is usually negative. The small lymphocytes of the non-neoplastic milieu are predominantly T-cells [118].

B.2 Mixed cellularity Hodgkin's lymphoma

Morphologic considerations: The neoplastic infiltrate is predominantly diffuse. Diagnostic Reed-Sternberg cells predominate as the neoplastic cell; some lacunar Reed-Sternberg variants may be present. The non-neoplastic milieu is composed of small lymphocytes, histiocytes, plasma cells, eosinophils, and neutrophils [118].

Immunophenotype: See nodular sclerosis Hodgkin's lymphoma.

B.3 Lymphocyte depletion Hodgkin's lymphoma

Morphologic considerations: Non-neoplastic milieu cells are relatively depleted and fibrosis and necrosis may be prominent. Diagnostic Reed-Sternberg cells and bizarre variants are plentiful [118].

Immunophenotype: See nodular sclerosis Hodgkin's lymphoma.

B.4 Lymphocyte-rich classical Hodgkin's lymphoma

Morphologic considerations: In contrast to nodular lymphocyte-predominant Hodgkin's lymphoma, the infiltrate in this entity is diffuse. There may small lymphocytes in the infiltrate; plasma cells, eosinophils and neutrophils are infrequent. Diagnostic Reed-Sternberg cells (rather than L&H cells) may be found but are infrequent. Lacunar cells may also be found.

Immunophenotype: See nodular sclerosis Hodgkin's lymphoma.

12.10NHL Molecular Panel Summary

The histopathological and immunophenotyping studies and standard cytogenetics analyses are the first steps and Gold standard for laboratory diagnosis of NHL and determine the type of the molecular tests needed for further analyses.

Mantle Cell Lymphoma (t(11;14)(q13;q32)): If it is indicated by pathological and immunophenotyping studies or cytogenetics studies show t(11;14) (70%), FISH for BCL1/IgH fusion gene(90%) will be followed, while PCR detects about 30-60% of the rearrangement.
Follicular Lymphoma (t(14;18)(q32;q21)): Aberration of BCL2 expression is found in 90% of FL, and t(14;18) is found cytogenetically in 75% of the FL cases. FISH for BCL2/IgH would confirm the diagnosis.
Anaplastic Large Cell Lymphoma (t(2;5)(p23;q35)): ALK immunophenotyping analysis still is the best for ALCL diagnosis. RT-PCR or DNA PCR is the method of choice for confirmation of NPM-ALK fusion transcript. FISH for ALK is also available to detect rearrangements involving ALK.
Burkitt Lymphoma (8q24): If Burkitt is diagnosed by pathological studies and/or 8q24 abnormalities are found in cytogenetics studies, FISH for c-MYC is the best to confirm rearrangement of c-MYC at this time.
NHL with Deletion of 6q: It is known as one of the most common chromosome abnormalities in NHL patients without t(14;18). It is present in about 7-14% of NHL and ALL patients. It could be sole change but most time as secondary changes. Recent studies revealed that del(6)(q25q27) in NHL and del(6)(q21q23) in ALL. FISH or PCR-based polymorphism studies have been shown to be more sensitive than standard cytogenetics to detect deletion of 6q. Some studies suggested that patients with del6q are shorter survival, but it is still a subject of debate. It might be necessary to study NHL patients with either FISH or PCR for deletion of 6q, and to distinguish two types of 6q deletions in patients del(6)(q25q27) vs del(6)(q21q23).

Frequency of 14q+ in NHL patients: t(14;18)(q32;q21) > t(8;14)(q24;q32) >t(11;14)(q13;q32)

Principal Chromosomal Abnormalities Characterized at the Molecular Level in NHL

Genes Involved	Abnormality	Incidence/histological subtype	Molecular Detection	Vysis
TAL1/TCRα/δ	t(1;14)(p32;q11)	1-3%	Southern	
TCL1/TCRα/δ	inv(14)(q11q32)	<1%	FISH	
BCL1/IgH	t(11;14)(q13;q32)	MCL	RT-PCR/FISH	available
BCL2/IgH	t(14;18)(q32;q21)	FL>DLCL	PCR/FISH	available
BCL3/IgH	t(14;19)(q32;q13)	?	?	
BCL6/IgH	t(3;14)((q27;q32)	DLCL>FL	Southern FISH	
c-MYC/IgH	t(8;14)(q24;q32)	Burkitt	FISH	available for c-MYC
NPM/ALK	t(2;5)(p23;q35)	ALCL	RT-PCR	available for ALK
PAX5/IgH	t(9;14)(p13;q32)	plasmacytoid	FISH Southern	
TCL1/ TCRα/δ	inv(14)(q11q32) t(14;14)(q11;q32)	T-PLL	FISH	

ALCL: anaplastic large-cell lymphoma aka Ki-1 lymphoma with CD30 (Ki-1) antigen
MCL: mantle cell lymphoma
DLCL: diffuse large-cell lymphoma

FL: follicular lymphoma
T-PLL: T cell prolymphocytic leukemia

13 OBTAINING, HANDLING AND STORAGE OF CLINICAL SAMPLES

Biological samples collected in this study fulfill clinical as well as research objectives and therefore must be handled according to standard protocols for the identification and processing of clinical samples.

13.1 Universal Precautions

In the United States, Universal Precautions are federally mandated and strict adherence to published rules must be in effect. This is applicable to all specimens of blood, serum, plasma, blood products, vaginal secretions, semen, cerebrospinal fluid, synovial fluid, pleural fluid, peritoneal fluid, pericardial fluid, amniotic fluid, and concentrated HIV or HBV viruses. Any specimen of any type which contains visible traces of blood should be handled using Universal Precautions. Barrier protection must be used to prevent skin and mucous membrane contamination from specimens which require Universal Precautions. These barriers include gloves, gowns, laboratory coats, face shields or mask and eye protection, mouth pieces, resuscitation bags, pocket masks, or other ventilation devices.

Universal Precautions will be mandated for all procedures performed in JCML and by JCML staff. Further, JCML will recommend Universal Precautions be performed by participating clinical coordinators and hospital staff to the extent that they are compatible with Chinese standards of practice.

14.2 Handling and storage

Blood and bone marrow will be immediately transported to JCML from collection sites. All samples will be transported at ambient temperature or on ice in the summer and should be analyzed while fresh. The International Committee for Standardization in Haematology (ICSH) defines a fresh blood specimen as one processed within four hours after collection. If the sample(s) are not fresh, specimen stability for each test will be considered when interpreting results. For example:

> The hemogram parameters - RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, and MPV - are stable (+/- 5%) for up to 24 hours after collection. The total WBC is stable (+/- 5%) for up to 12 hours after collection. The stability of the total WBC decreases to +/- 7% at 24 hours after collection.
>
> The WBC Differential parameters - NEU, LYM, MONO, EOS, and BASO - are stable (+/- 10%) for up to 12 hours after collection. An increase in false positive Suspect Population Flags may be seen on samples processed less than 30 minutes after collection time or more than 4 hours after collection time.
>
> Stability studies conducted at Abbott indicate that specimens exhibit increased stability

when they are stored at room temperature rather than in a refrigerator.

The stability of capillary specimens collected in microtainers may vary depending on the microtainer manufacturer. Refer to the manufacturer's package insert for stability claims.

All stored samples will be labeled with the patient's ID code, accession code, date, volume and technician's name. Any material specific information can be accessed via the accession code within the research database.

Blood Samples	Storage
In EDTA	None
In heparin	EBV transformed cell line will be frozen and stored in liquid nitrogen
Serum	-80 C freezer

Bone Marrow Samples	Storage
Slides from touch preps	Desiccated -80 C freezer
Slides from smears	Desiccated -80 C freezer
Paraffin block	Room temperature
Slides from block	Room temperature
Slides - banded analysis	Desiccated room temperature
Cell Pellets - FISH	-20 C freezer
Slides - FISH	Desiccated -20 C freezer
RNA	-80 C freezer
DNA	-80 C freezer
Frozen cells	Liquid nitrogen

14 BIOLOGICAL SAMPLING

14.1 Blood

A Introduction

Blood will be drawn on all study subjects except controls. For in-patients being worked up for BP, AA, MDS or AML blood will be taken by venipuncture as follows: A total of 5 ml of blood will be drawn: 2 ml in EDTA for CBC and 3 ml in heparin for lymphocyte immortalization. When possible manual slide smears will be obtained as well. For subjects being worked up for NHL an additional 3 ml will be collected in a serum collection tube for serology. In any case where a lymphoid neoplasm cannot be ruled out, 3 ml will be collected for serology.

B Sampling Objectives

Blood will be drawn for CBC and lymphocyte immortalization on all study subjects except controls. Additional blood serum samples will be taken for serology on NHL cases or cases where NHL cannot be ruled out at the time of recruitment.

C Sampling Procedures

For routine blood drawing procedures the preferred site will be the anticubital vein. The site should be sterilized using 70% isopropyl alcohol and allowed to dry prior to taking the specimen. A tourniquet may be applied 3 to 4 inches above the site to distend the vein prior to venipuncture.

For blood drawing protocols see Appendix A.

14.2 Bone Marrow

A A Introduction

Bone marrow biopsy and aspiration will be performed on subjects enrolled in the Disease Progression Study who are suspected of meeting criteria for diagnosis with severe AA, MDS, AML or BP.

B B Sampling Objectives

Biopsy and aspirate material will be collected for morphologic and cytologic analysis, histochemical staining, immunophenotyping, cytogenetic (e.g. banded chromosome analysis, FISH, m-FISH) and molecular analysis of clonal lesions, and tumor EBV status. The results obtained from these procedures will support differential diagnosis and follow-up of subjects' disease state as well as study classification objectives. In addition, a small amount of bone marrow may be frozen for future analysis of bone marrow phenotype and growth characteristics.

C C Sampling Procedure

1. Ideally, the site for the bone marrow biopsy should be the posterior superior iliac spine. The location of the right or left posterior superior iliac spine should be located, marked by making an indentation in the skin with plastic needle guard and sterilized with iodine solution, following which sterile conditions should be employed.

2. The biopsy site should be sterilized by washing three times with a gauze soaked in iodine solution. Start at the center of the biopsy site (i.e. over the posterior superior iliac crest), and move out in a circular pattern for 15 to 20 cm. Following sterilization, wash the biopsy site once with an isopropanol-soaked gauze. Non-sterile "floor exam" gloves may be used for the sterilization procedure but need to be replaced with sterile gloves

immediately after preparation of the site. The sterile site should be draped with sterile drapes.

3. The dermis, subcutaneous tissue and periosteum should be properly anesthetized with 1% xylocaine over the posterior superior iliac spine so that there is enough anesthetized area to perform two separate biopsy procedures, should this prove necessary. And an small incision is made over the posterior superior iliac spine.

C.1 Bone marrow aspirate

1. The biopsy needle is introduced into the marrow cavity and EDTA, coverslip and heparinized samples of bone marrow aspirate prepared according to the BMB-Aspiration Flow Chart.

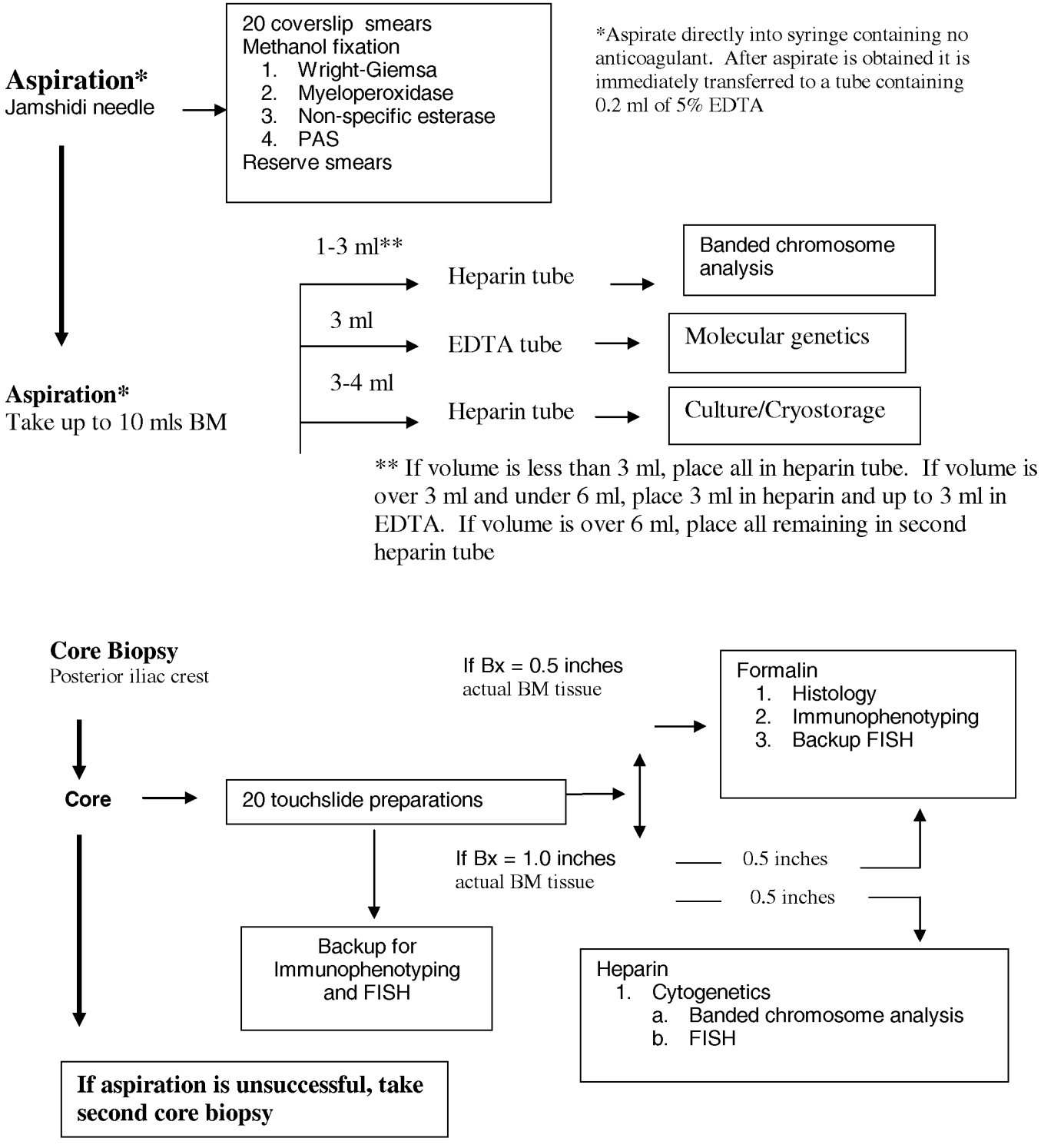
2. If it is not possible to obtain a bone marrow aspirate then two core biopsies should be obtained and allocated according to the Bone Marrow Biopsy-Aspiration For Collection Protocol see Appendix C or Figure 15.1 Below.

C.2 Bone marrow core biopsy

1. The core biopsy should normally be obtained through a second bore hole or the same hole using a Jamshidi biopsy needle after the aspirate to prevent procurement of an artifactually diluted aspiration sample. A 2.0-2.5 cm core should be obtained and the needle removed by applying steady backward pressure and rotating in a clockwise and counterclockwise direction.

2. Touchslide preparations should be made and the core biopsy allocated according to the Bone Marrow Biopsy-Aspiration Flow Chart

Figure 15.1 - Bone Marrow Biopsy - Aspiration Protocol



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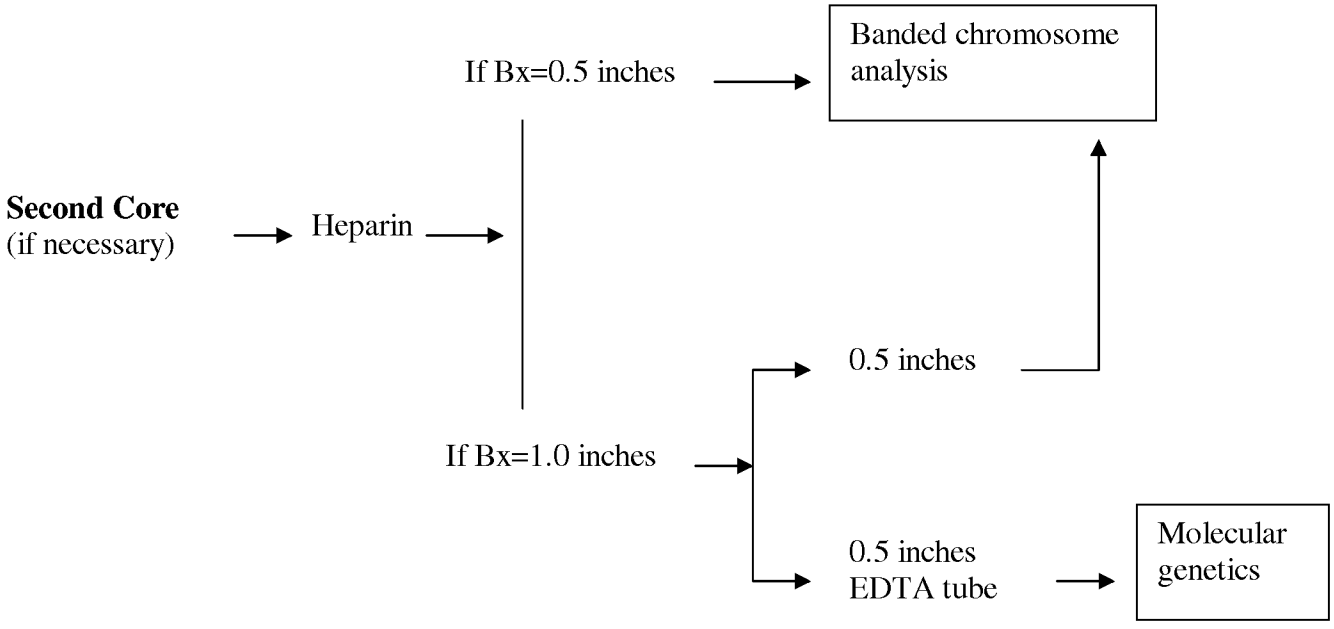
Bone Marrow Biopsy-Aspiration Protocol
(Continued)



15.3 Lymph Node and Tissue Biopsies

Principle

High-quality sections for routine light microscopy are necessary, but not always sufficient, for the interpretation of lymph node or bone marrow biopsies. Immunophenotypic and genetic studies are often required for the proper diagnosis and classification of a hematopoietic neoplasm. Adequate fixation and timely and appropriate technical handling of the tissue are, therefore, even more important than with other specimens.

A Gross Examination and Specimen Processing

Once a resected lymph node specimen is received, its size, shape and capsule situation will be described. It will then be transversely sliced into uniformly 3mm-thick sections. The cut surface will be examined carefully and the gross appearance documented in detail. Then at least 2 representative sections will be submitted for routine fixation immediately (See Figure 15.2). Bone marrow trephine core biopsies will not be sectioned unless actual bone marrow tissue is 1 cm in length. See Bone Marrow Biopsy Flow Chart (Figure 15.1).

15 HEMATOLOGY

15.1 Instrumentation

The CELL-DYN 3700CS System will be used routinely at JCML. It is a multi-parameter, automated hematology analyzer with a manual Closed Sampler designed for in vitro diagnostic use in clinical laboratories. (See Appendix D).

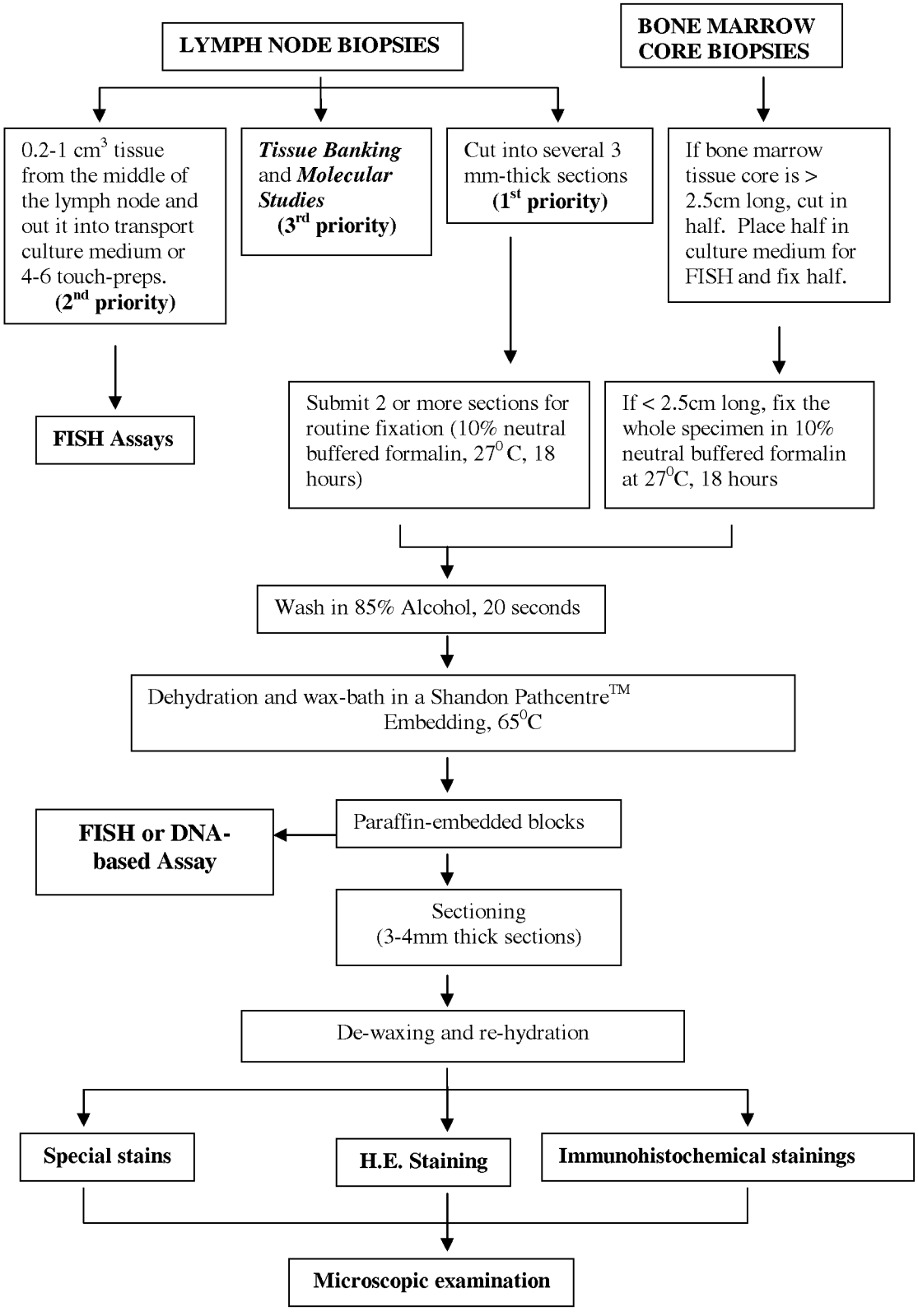
15.2 Procedures

The CELL-DYN 3700CS System generates the following hematologic measurements on EDTA-anticoagulated whole blood:

WBC - White Blood Cell or Leukocyte count
NEU - Neutrophil absolute count
%N - Neutrophil percent
LYM - Lymphocyte absolute count
%L - Lymphocyte percent
MONO - Monocyte absolute count
%M - Monocyte percent
EOS - Eosinophil absolute count
%E - Eosinophil percent
BASO - Basophil absolute count
%B - Basophil percent
RBC - Red Blood Cell or Erythrocyte count
HGB - Hemoglobin concentration
HCT - Hematocrit
MCV - Mean Corpuscular Volume
MCH - Mean Corpuscular Hemoglobin
MCHC - Mean Corpuscular Hemoglobin Concentration
RDW - Red Cell Distribution Width
PLT - Platelet or Thrombocyte count
MPV - Mean Platelet Volume
PDW - Platelet Distribution Width
PCT - Plateletcrit
RETIC % - Reticulocyte Percent
RETIC ABS - Reticulocyte Absolute
IRF - Immature Reticulocyte Fraction

In addition, Wright's-stained peripheral blood smears will be made routinely for use as a back-up in the evaluation of peripheral blood morphology.

Figure 15.2- Flow Chart for Processing of Lymph Node and Tissue Biopsies



16 IMMUNOHISTOCHEMISTRY

16.1 Processing of Samples

Immunochemistry and histochemistry will be performed on 3-4 micron paraffin sections of lymph node, tumor and bone marrow. Sections will be prepared in the Department of Pathology, Shanghai Tumor Hospital, Fudan University Medical Center. Deparaffinization and immunostaining will be routinely performed using the Ventana Discovery System at JCML.

17 FISH

17.1 Introduction and Principle

Fluorescence *in situ* hybridization (FISH) is a DNA-based molecular cytogenetic technique with high resolution for detecting specific recurring acquired abnormalities in leukemias and lymphomas and cryptic chromosome changes that otherwise might not be detected by standard cytogenetic analysis. FISH analyzes both dividing and non-dividing cells, and plays unique roles in monitoring residual tumor cells in follow-up patients. Both studies are complementary to each other. They provide important diagnostic and prognostic markers and guidance for the treatment for hematological malignancies. FISH of tissue sections and cell preparations will be automated using the Ventana Discovery System (Appendix F).

18 CHROMOSOME G-BANDING ANALYSIS

18.1 G-Banding Procedure for Lymph Node and Bone Marrow Preparations

G-banded chromosome or standard cytogenetic analysis studies of the morphology of human chromosomes is considered the gold standard in clinical cytogenetics. After pre-treatment with a proteolytic enzyme such as trypsin, chromosomes may be stained with a Romanovsky Stain (e.g. Giemsa, Leishman, or Wright Stain) to produce alternating light and dark bands along the length of each chromosome. The stain binds preferentially to specific parts of the chromosome, with dark and light staining regions corresponding to the base pair composition of each region. Dark bands are A-T rich and contain relatively few active genes, while light bands are G-C rich and contain many active genes. Each chromosome has a characteristic G-Band pattern, which aids in the identification of whole chromosomes, translocated parts of chromosomes, and rearrangements within chromosomes. G-banding analysis will be routinely performed on lymph node and bone marrow preparations in this study. (For individual protocols see Appendix G).

18.2 Preparation of Slides for Cytogenetic Studies

A Overview

The method in which cytogenetic cell suspension slides are prepared profoundly affects the accuracy and expediency of cytogenetic analysis. Accordingly, a thorough appreciation and understanding of the mechanisms and requisite techniques necessary in procuring optimal metaphase slide preparations is critical. Our culturing techniques focus on capturing a high yield of metaphase spreads at band levels exceeding the 650 band stage of chromosome condensation. Thus optimal slide preparations should contain adequate numbers of mitoses with elongated, straight chromosomes. These should be minimally overlapped, and have good morphology (contrast) which will result in consistently informative staining / banding patterns at all band lengths. The intent of slidemaking is to consistently and expediently procure the highest possible ratio of optimal, elongated metaphase spreads on each individual preparation. The Thermatron slidemaker with controllable humidity and temperature will be used to make slides with consistent good quality. This standardization is critical in a climate such as that encountered in Shanghai. For protocol see Appendix G.

19 MOLECULAR GENOTYPING PROTOCOLS

19.1 Isolation of Genomic DNA and RNA from Lymph Node, Tumor (lymphoid) or Bone Marrow Tissue

Principle: The protocol to be used is generated for purification of genomic DNA from up to 25 mg tissue. The quality of the DNA depends on the preservation of the starting material. The typical yield of the DNA is 10-40 ug although it may vary according to the type of the tissue. Larger amount of starting material require increased amount of solutions used in the procedure. RNase might be need to remove RNAs from tissues like liver and kidney, replace Step 3a with Step 3b in the following protocol. The protocol for isolating RNAs combines the stringency of guanidine-isothiocyanate lysis with speed of silica-membrane purification. (See Appendix H)

20 IMMUNOASSAYS FOR HEPATITIS B (HBV), AND HEPATITIS C (HCV)

The IMx System (Abbott) will be used to perform HBV and HCV immunoassays as part of the study protocol and HIV1/2 assays if requested by attending physicians. The enzyme immunoassays procedures use a coated submicron microparticle as the means by which the analyte to be measured is captured for analysis. Assays which use this method are called Microparticle Enzyme Immunoassays (MEIA). The IMx System uses a number of different assay modules, each containing several assay files. The assay modules that contain the HBV and HCV assay files will be run for each test. HIV1/2 will only be run if requested in clinical support of a diagnosis. Each assay requires a minimum of 150 ul serum. The analytes for HIV1/2, HBV, and HCV are HIV1/2 antibodies, HBV core antigen, and HCV antibodies. Assay-specific controls as supplied by Abbott will be used to monitor the accuracy and precision of the IMx System, reagents, and assay results.

20.1 Procedure

See Appendix I

21 DETECTION OF LATENT EBV INFECTION IN NHL TISSUE SECTIONS

The two assays most frequently employed in the determination of latent EBV infection are immunohistochemical identification of LMP-1 surface membrane expression and demonstration of EBV EBER nuclear gene transcripts by in-situ hybridization. Because LMP-1 expression is variable and unpredictable, we have chosen to measure EBER.

21.1 Procedure

Fluorescein-conjugated EBV Oligonucliotides will be utilized for the detection of latent EBV infection in lymphoid tumors by in-situ hybridization on formalin-fixed, paraffin-embedded tissue sections. The probe hybridizes to abundantly expressed **Epstein-Barr Virus**-encoded RNA (**EBER**) transcripts which are concentrated in the nuclei of latently infected cells. In situ hybridization will be performed using the automated Discovery system.

22 COLLECTION OF BUCCAL SWABS/ IMMORTALIZATION OF PERIPHERAL B LYMPHOCYTES

22.1 Principle

Germ line DNA will be obtained from subjects either by collection of buccal swab or immortalization of human B lymphocytes isolated from peripheral blood. This provides a renewable source of subject DNA for analysis of genetic polymorphisms or mutations.
Immortalized lymphocytes can be frozen and stored for long periods of time or cultured and harvested. (For protocol see Appendix J).

A Database

A separate database will be maintained for immortalized cell lines that does not contain subject identifiers or residual linkages with other databases. This will enable independent correlation of clinical and research data with immortalized cell lines. This database, along with immortalized cell lines, will be preserved at the end of the study and will become the intellectual property of Fudan University.

23 EXPOSURE ASSESSMENT STATEGY AND EXPOSURE MONITORING

23.1 Exposure Assessment Approaches.

A Introduction

The tiered approach discussed in this document provides a means of meeting the exposure assessment needs of the family of planned epidemiology studies; different levels of exposure assessment information are needed for different phases and aspects of the various studies. Each tier builds upon the information and data used in prior levels. The tiers outlined below are directed toward the disease progression study and other case-control aspects, but also form a base for the molecular epidemiology study. See Figure 25.1 (following) for an overview of the exposure assessment process. In this section, we provide information the exposure assessment approaches for:

1. Ordinal range exposure to benzene
2. Quantitative assessment of work history benzene exposures
3. Pattern of benzene exposure assessment
4. Potential confounding exposures

A fourth tier (detailed personal and biological exposure monitoring) is discussed in the Molecular Epidemiology protocol. These tiers are not intended to be rigidly sequential. In many cases, especially during initial facility reviews, information for several of the anticipated assessment levels will often be assembled, in order to minimize repeat site visits.

For the disease progression study, the initial assessment goal is to sort study subjects (cases and controls) into one of three categories: likely unexposed, uncertain, and exposed. For the exposed subjects, they will subsequently be assigned to ordinal benzene exposure categories (as discussed below). Then, follow up assessment (tier 2) will be used to clarify the uncertain exposure category and to further quantitate the work history exposures for the benzene-exposed subjects. Tier 3 will be used to determine the exposure concentration-time patterns of the benzene-exposed subjects.

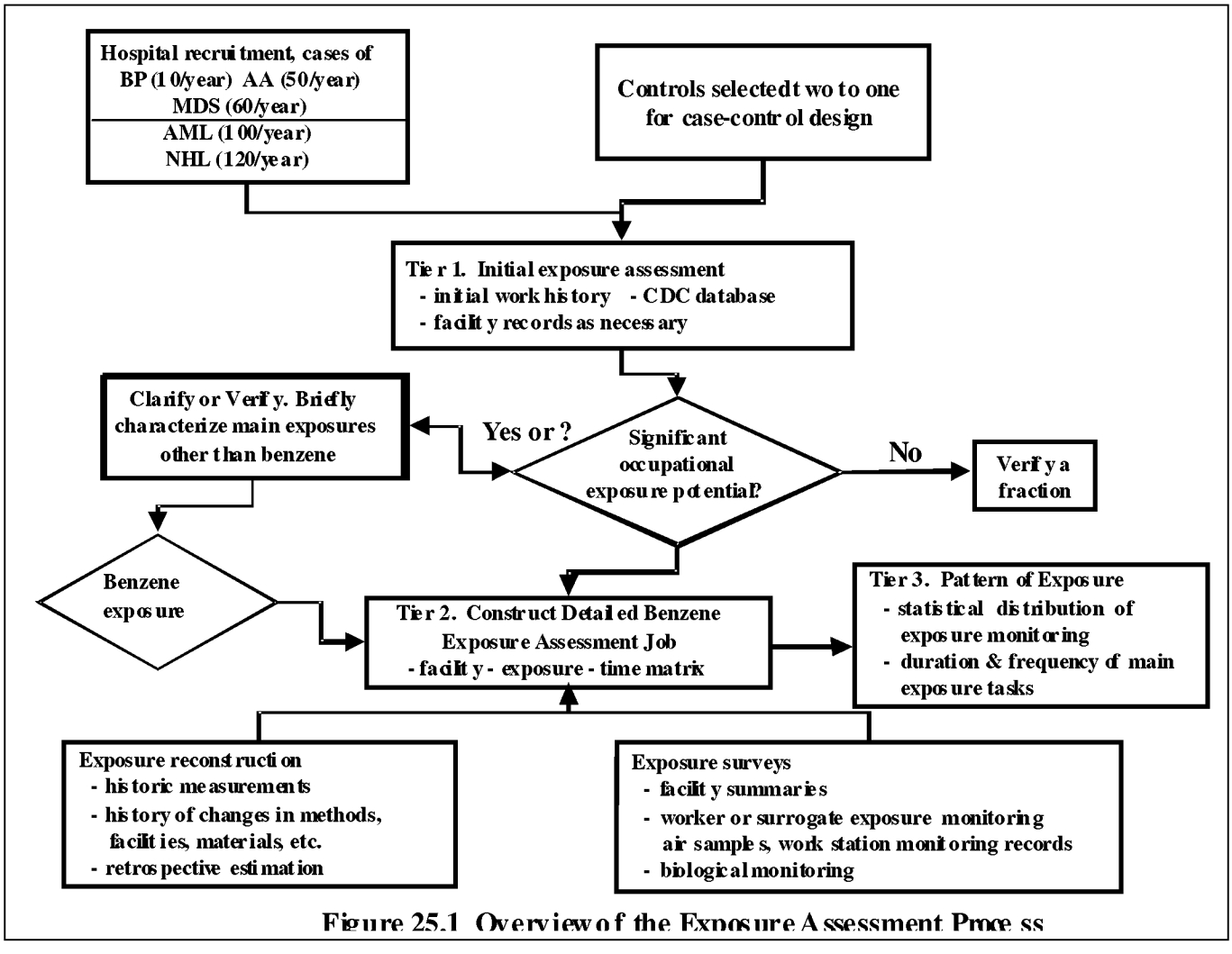


Figure 25.1 Overview of the Exposure Assessment Process

B Tier 1. Ordinal Range Exposures to Benzene

This provides the first stage of the assessment strategy, and will be used as a component of the exposure assessments for all of the study exposure assessment aspects. The main goal for the Tier 1 assessment stage is to assign benzene exposed subjects to ordinal ranges, for each major segment of their work history. The ranges for the categories, such as 0 to 1 ppm, 1 to 10 ppm, 10 to 50 ppm, and > 50 ppm, will be set following further evaluation of the Shanghai industries' exposure data.

Aspects discussed in this section include:

- Study enrollment questionnaires and initial work histories

- Shanghai Municipal Centre for Disease Control and Prevention (SMCDCP) monitoring database queries and data summaries
- Assessment of exposure to chemicals other than benzene
- Facility reviews and workplace record use
- Exposure classification process
- Data time trends

Study enrollment questionnaires will be used to initiate the work history information for this tier and subsequent tiers of the exposure assessment. Subjects will be entering the study from a broad range of employment, industries and job categories in the Shanghai area. The Shanghai Municipal Centre for Disease Control and Prevention (SMCDCP) database will be queried for information about benzene and other exposures for each subject's work location (if employed in an industry covered) in order to classify study personnel according to factory type and occupation. Factories or jobs not matching on "known benzene exposure" criteria from the database query will be set aside for further review and assignment. Our experience to date reveals that this group is much higher than originally anticipated. Thus, we have supplemented this strategy with a review of the Chinese literature, the Yang-Pu database, real time monitoring in select facilities, and simulations/recreations of past conditions.

For other chemical exposures, the intent at this stage is simply to record the identity of other major chemical exposures for the subject. The SMCDCP database may have a lower information yield for chemicals other than benzene since many other chemicals do not have disease registries and the long standing regulatory requirements such as are established for benzene poisoning.

Work history information collection will start with recruitment of the subject. An interview will be conducted to determine the subject's employment history, and as much as possible of the job positions held. The main objective of the initial work history interview is to establish the type of industry, the work location, and to the extent possible, main jobs held. We do not expect to use interviews to gather worker's self reported exposures, as these are known to be often unreliable (Frischi 1996) and better information sources, such the CDC database and factory records, will be available. The hospital staff who recruits study subjects will conduct this initial interview. A standardized interview data recording form and instruction in its use will be provided. The exposure assessment staff may complete follow-up interviews using a more detailed questionnaire, following their review of the initial interview results. Follow-up will also be completed the initial interviews that were classified as having no significant occupational exposure (e.g., administrative office only jobs). If a subject is incorrectly assigned in a 10% sample, the classification process will be reviewed and modified. Sequential sampling methods will be applied to assure that no more that 1% of the remaining "unexposed" subjects are incorrectly assigned.

For many subjects, workplace records (rather than follow-up interviews) will be the primary and preferred source for details about their work history. As a general rule, most workers in Shanghai tended to stay with the same work location for a career. This work history stability will enhance the role of facility records for work history and exposure history development. However, in cases where records are unavailable or are inadequate (for example, small or closed employment sites), follow-up interviews will be the main information source. The work history

records for this project will be coded with regard to the source(s) of the information for the work history (for example, subject interviews, other interviews, written records, combinations of sources.) For small sites, closed sites, or for non-industrial employment, techniques discussed in the following retrospective assessment section will be used. These include read-acrosss to most similar jobs, or collecting current information and adjusting to represent important changes in exposure potential.

Following assembly of the work history, the exposure assessment staff will construct the subject's exposure history. The first objective will be to classify the subject as a) likely exposed to chemicals, which may or may not include benzene b) unexposed to chemicals above non-occupational background or c) requires further investigation to adequately classify the subject's exposure potential. This classification will be completed by a combination of professional knowledge and information from the SMCDCP database and other sources. Going back in time (beyond approximately 1987 and the start of the database) will require much further investigation of written sampling records and facility information. See section 25.7 Retrospective Exposure Assessment Approaches for further discussion.

For the "exposed" subjects, the initial exposure characterization for the subject's work history will be completed to the extent possible based on the SMCDCP database (and other data sources as mentioned above) for current and recent past work locations and job assignments. Measured exposure data in the database were largely obtained using the SMCDCP traditional short-term breathing zone samples that were taken at specified work locations. Part of the exposure assessment development will be relating these to personal exposure. (Initial indications are that there may be a systematically "low" bias in the IPHS database, however, this is still under evaluation).

One of the objectives of facility visits and additional monitoring (concurrent using both personal monitoring and traditional short-term breathing zone measurements) is establish how the available data represents the subjects' exposure. This additional monitoring will generally cover workers in the same job assignment as held by the subject whose exposure history is being developed. To translate the short-term breathing zone measurements into personal full shift exposure estimates, a "task-time weighted average" approach will be applied (Smith TJ, et al 1991), in conjunction with an exposure zone approach (Corn and Esmen, 1979), if feasible. See Section 25.7 for further discussion of statistical analysis of the exposure data. The exposure data (full shift personal samples, short-term breathing zone samples, and possibly partial shift task-specific personal samples) will also be used for probability distribution analysis using standard methods (Mulhausen and Damiano 1998) or via Monte Carlo simulation methods (Thompson et al, 1992). See Section D. Tier 3. Assessing the Pattern of Exposure (below) for further discussion.

For other chemicals identified as present and involving occupational uses in the workplace, only their presence will be noted. At present, for the disease progression studies, there is no plan to quantitatively evaluate the extent of other exposures.

This ordinal ranking process for benzene will be used as a starting point for the exposure assessments supporting the studies. The ordinal ranking will also be part of the historic exposure estimating process. However, how far back retrospective assessment can be confidently

accomplished requires further investigation. The extent and nature of workplace design changes, work practice changes, changes in materials used, and work hour changes are a few examples of factors to resolve during the field phases of the exposure assessment project.

C Tier 2. Quantitative Assessment of Work History Exposures

Individual job-location exposure records existed for the factories evaluated in the study feasibility investigation. Additionally, the SMCDCP has regulatory authority for access to factories and for conduct of exposure measurements. (However, our experience to date is that we cannot access as many factories as originally envisaged, since IPHS did not assign dedicated staff to this project as originally plan). Steps in assembly of information to develop the individual subject's work history-based quantitative benzene exposure assessments include:

1) Work history initial development. This has been discussed in Section B above. For cases and controls, current factory and current job information will be obtained from an initial questionnaire. The SMCDCP database will then be queried for an initial profile of the factory and exposure potential. From the SMCDCP system, a classification into exposure categories will be completed. Note that for subjects classified as not exposed to benzene, based on information from the database, no further work on the exposure history will be routinely completed. Again, a statistical sample will be further evaluated as a quality assurance process.
2) Work history completion. For subjects for whom a complete and detailed work history is needed, factory records and interviews will be used to expand the initial work history. Interviews may be used for coworkers or supervisors in the facility, as well as for the study subject. Again, the record will be coded with regard to the information source. For each job held by the subject, the nature of the work performed and the frequency/duration of work with benzene-containing materials and/or in benzene-contaminated areas will be established. This information on the tasks will be used with task-specific exposure data in a task-time weighted average model to develop an estimate of the associated job exposure. Details on short duration, high exposure activities (possibly infrequent) will be sought, as will be the information on the more routine tasks. If sufficient data (air concentration, task duration, and task frequency/intermittence) are available, a probability distribution of the estimated task exposure will be developed.
3) Exposure monitoring records. The SMCDCP database will be queried regarding exposure data for the relevant jobs and factory according to the information gathered in steps 1 and 2. At present, it seems that the SMCDCP database is structured so that each year's monitoring data are in a separate file. One early objective is to consolidate the records so that multi-year summaries and analyses of the data may be more easily and rapidly completed. This may require migration of the database to different software. Additional exposure monitoring records from factories will be assembled, entered into the database (coded appropriately to track its origin and descriptors) and summarized to supplement the existing SMCDCP records. This will be essential for more historic job exposure estimates. The data will be subject to the time-trend and statistical analyses previously discussed (Section 25.1 B.) The data assembly will be structured to provide long-term average exposure estimates, variability of exposure information and specific

job activity related exposure data. The job-activity exposure data are particularly of value in exposure reconstruction when the frequency or duration of the activity may have differed, or was performed by someone in another job category. (Note: the IPHS database will be of less utility than originally anticipated; other data sources mentioned above will be used to supplement exposure estimates).

If data are sparse for a given facility, use of data from other factories within the industry segment will be considered, if several conditions are met. Some of those factors to be considered include: the similarity of: physical structure, layout, equipment, controls (enclosure, ventilation, other), work assignments, materials used and material composition. The decision on application of data from other facilities will be made by personnel who have visited the facilities or who have other substantive rationale on which to establish the similarity.

Initial site characterization efforts will focus on the work areas relevant to the subject. This should cover the work locations where all the initially known potential exposure activities (even if infrequent) occur. A walk-through inspection and screening level measurements for benzene (see Section 25.3) will be completed. The objective of this review is to identify the locations from the area monitoring database that are relevant to the subject, and identify any significant gaps in coverage. The gaps will be resolved via additional, new monitoring.

4) New monitoring. SMCDCP network and/or SMU personnel will compete additional monitoring to develop exposure profiles for facilities where further data are needed to characterize current exposure or to support estimation of past exposure. Note that the SMCDCP has regulatory authority to undertake the monitoring. The surveys will be either targeted to specific tasks/activities and/or randomly completed for the job assignments relevant to the subject's work history. Targeted surveys will provide data for specific data gaps. The random data will support developing statistical projections of long-term average exposures, and statistical estimates of exposure variability.
5) Dermal exposure potential. We will evaluate the potential for dermal contact and its relative importance compared to inhalation exposure. We will use a "range finding" table (too be developed) that provides estimates (from conservative modeling) of the absorbed dose resulting from: assumed ranges of a) extent and frequency of skin contact and b) the potential benzene-content of the materials used. Its use will depend on the ability to estimate the potential dermal contact pattern and to understand the materials contacted for the subject being assessed. Initially, these may be rather rough, uncertain and conservative assumptions. They can be used to decide, based on the estimated inhalation potential, whether or not further consideration of the dermal contribution is merited. If so, efforts to better quantitate (e.g., via better characterization of contact and materials or refined modeling) the dermal exposure will be considered. However, without use of unusual control measures or routine use of reliable respiratory protection, we expect elevated dermal exposure will also involve substantial inhalation exposure. If further dermal assessment is undertaken, the benzene content of the materials contacted becomes an important parameter to elucidate.
6) Reconstruction and monitoring. Reconstructing certain exposure scenarios will be undertaken, if necessary to fill key data gaps. Then, measurement will provide data for historic exposure assessments. Personnel involved in the reconstructed work should be protected from overexposure according to current good operating practices. See Section 25.7 (Retrospective Exposure Assessment Approaches) for further discussion.

7) Review of Chinese data for industrial segments with a larger percentage of cases and controls.

Details on monitoring methods are provided in Sections 25.3, 25.4, and 25.5.

23.2 Blinding

Blinding (intentionally keeping the exposure assessment staff from knowing the subject's case/control status) reduces the likelihood of biasing the extent of effort and the assigned results in the exposure assessment. In these studies, blinding will be maintained when necessary and where possible. For the Disease Progression study, blinding as to disease status should be maintained. However, once the initial stratification into exposed, unexposed, or uncertain has been done, access to the "exposed" or uncertain subjects may be needed to clarify work history and lifestyle/hobby aspects through subject interviews. At that time, blinding could be compromised. We think that this is not an overwhelming issue, compared to the benefits of improving the work history and consequently the exposure information. Nevertheless, steps will be taken to minimize compromising blinding, and/or understand and control for possible bias if blinding is compromised.

The exposure assessment staff will be briefed with regard to the potential biases if blinding is compromised. The staff will be instructed not to intentionally seek to learn the subject's case/control status. The work history information form will have a block for the exposure assessor to indicate if they learned or have strong suspicion of the subject's status. This code may be used in the epidemiology analyses as a "confounding" variable. Another approach will be to complete follow-up interviews for a sample of subjects who have complete, records derived work histories, and where the follow-up interview indicates blinding was compromised. Then, a comparison may be made on the differences in exposure assessment from the two variations on the work history. The third (but not favored) alternative would be to have another exposure assessment team member complete the exposure assessment. However, our preference will be to have the staff member most knowledgeable about the facility, exposure data, tasks, and other exposure information complete the assessment, even if possibly aware of the subject's status.

The Molecular Epidemiology study (Protocol II) is prospective, so blinding of the exposure assessment staff is not a factor.

23.3 Exposure Assessment Air Sampling and Analytical Procedures

Passive Sampling Organic Vapor Monitoring. Passive organic vapor monitors (POVM) will be the primary collection method for new surveys of air concentrations. These will be used for both full-shift breathing zone samples, and for monitoring during key tasks to establish their contribution to total exposure. For these studies, we expect to select and use primarily 3M 3500 or SKC 575-001 devices. However, 3M 3520 devices will be used where high exposures (e. g. over 50 PPM) are anticipated since these devices have higher capacity and a backup section to evaluate if overloading occurred. Unique location and job identification codes will be used for tracking the samples and data in the project. Each sample collected will have a field data record in the study database. See Figure 25.2 (following page) for an example of a field data recording

form that will be modified to meet the Shanghai study needs. Computerized forms will be used to the extent possible to minimize both data transfer time burdens and potential transcription errors. Surveys will also employ more traditional Chinese exposure measurement techniques (i.e., short-term breathing zone monitoring using charcoal tubes and a calibrated air sampling pump) in order to establish analytical and survey equivalence.

Laboratory analysis will follow the procedure outlined in NIOSH Method 4000: "Toluene by diffusive sampling." The file is available at: http://www.cdc.gov/niosh/nmam/pdfs/4000.pdf. This laboratory method is also applicable (and is widely used) for benzene. Passive organic vapor monitors have been used in previous studies in China (Rothman 1996), and have been used by and analyzed at the SMCDCP laboratory. Additional information on laboratory procedures for analysis of samples collected by diffusive samplers is available in the UK HSE Method MDHS 88. *Volatile organic compounds in air. Laboratory method using diffusive samplers, solvent desorption and gas chromatography.*

A quality assurance process will be established, involving spiked and replicate analyses, with confirmation of a statistical sample of replicates by an independent laboratory, or via "round robin" testing in a standard Proficiency in Analytical Testing (PAT) program. See Section 25.6 for further discussion of the quality assurance plan for the industrial hygiene monitoring aspects of the project.

23.4 Exposure Assessment - Direct Measurements with UltraRAE PID for Benzene

A rapid screening measurement device for benzene will be used since it will allow on-site evaluation of benzene exposure potential, and thus give the exposure assessment staff immediate information on current benzene exposure levels. This will be of value in planning the work areas and activities to cover in a survey using laboratory methods, and will also help hone the field crew's observational skills. Generally, photo-ionization detectors (PID) are useful for screening measurements down to very low ppm levels of volatile organic compounds (VOCs), for example, petroleum solvent vapors, but are not selective and cannot give data for a specific compound in a mixed chemical environment. However, the UltraRAE (manufactured by RAE Systems, Sunnyvale, CA www.raesystems.com) has several innovations that enhance it as a benzene selective PID. It utilizes a lower energy lamp (9.8 eV) for benzene monitoring (benzene ionization potential = 9.25 eV). A 9.8 eV lamp discriminates against other hydrocarbons with higher ionization potentials commonly found in petroleum vapors yet will allow detection of sub-ppm levels of benzene. Benzene measurement also involves use of a patented tube that contains absorbent materials to remove most other VOCs, such as toluene, that will interfere with the benzene reading. An internal pump draws the air sample through the tube and the final result is shown on the display (after 30 to 75 seconds depending on the compound). These tubes also absorb humidity to allow for an accurate reading on the UltraRAE. The data-logging feature then records the date, time of monitoring, and the concentration. Calibration of the instrument with "zero air" and known concentration span gas takes a few minutes.

Condensed, step-by step instructions and the manufacture's instruction manual provide further information needed on calibration, maintenance and use procedures. Certified benzene span gas will be used for daily pre and post use calibration. Daily field monitoring logs and a permanent calibration log book for each instrument will be used to record the calibration.

Figure 25.2. Example Field Survey Data Recording Form

Company:_____ Date:_____
Address:_____ Subject File #:_____

Area/Work Section:_____
Worker Monitored:_____
Worker Job Title:_____
Job Description:_____

Exposure Assessor Name:_____
Temp:_____ Humidity:_____ Wind Speed:_____

Task Descriptions: (use reverse of form for additional tasks) Include materials/volume/temperatures
1._____
Frequency:..... Duration:.....
2._____
Frequency:..... Duration:.....
3._____
Frequency:..... Duration:.....
4._____
Frequency:..... Duration:.....

SAMPLE DATA	Sample ID #	Sample ID #	Sample ID #
Task(s) covered:			
Start time:			
Stop time:			
Total Time:			
Type:			
Strategy:			
Substance(s)			
RESULTS:			
Substance:			
Concentration:			

Calibration Information (if applicable)
Instrument ID#:_____ Calibration Gas ID Information:_____
Pre-Shift Result:_____ Post Shift Result:_____

Air Sampling Pump ID:_____
Pump Flow Rate Pre-Shift_____ Post-Shift_____

Production Conditions: Normal ☐ Low ☐ High ☐

Exposure Controls*:_____
Effectiveness: Good ☐ Moderate ☐ Poor/Ineffective ☐
* use reverse of form for details for different tasks.
COMMENTS:

23.5 Exposure Assessment - Field Monitoring and Information Collection Procedures

The US National Cancer Institute (Stewart, 1998) developed a series of job-specific exposure history interview forms that we will adapt for use in this study. The NCI questionnaires suggest a series of questions to elicit and record information about task duration, frequency, and exposure potential. The questionnaires will not likely cover jobs of relevance to the Shanghai studies, but will be used as starting points.

23.6 Exposure Assessment - Quality Assurance Plan

An industrial hygiene laboratory analysis and field sampling quality assurance detailed plan will be developed that covers the main points of the NIOSH quality assurance procedures given at: http://www.cdc.gov/niosh/nmam/pdfs/chapter-c.pdf
Aspects of this include:

- Appointed quality assurance coordinator (preferably not reporting to laboratory management)
- Sample tracking clerk
- Quality assurance for sample collection (e.g., calibration, data recording, shipping conditions)
- Laboratory measurement quality assurance (standard methods, reagent blanks, field blanks, blind samples, recovery studies, duplicates, calibration procedures)
- Inter-laboratory testing (proficiency verification)
- Instrument maintenance and calibration procedures, records
- Sample tracking
- Documentation and document retention procedures.

Also see Section 24.2 for discussion of quality assurance testing of air samples.

23.7 Exposure Assessment - Exposure Monitoring Statistical Plan

Several exposure metrics will be developed for the subjects in the disease progression study, including long-term average benzene exposure. That is, the typical exposures over the course of a week, or month, or year. There is also an objective to study the relationship of disease risk to the pattern and variability of exposure, as discussed in Section 25.1 D (above). This report section will briefly discuss the statistical rationale for a sampling plan to support their determination.

The available monitoring data will be evaluated for time trends via a sequential plot (Mulhausen and Damiano, 1998). If the plot shows a suggestion of time trends (e.g., positive or negative slope over several years, or seasonally cyclic variation), the data will be tested for significance of the trends by appropriate time intervals (e.g., quarters, year, 5 year or 10 year). If significant, the data will be used to generate time period specific estimates of exposure. Appropriate summary statistics (i.e., arithmetic mean, geometric mean, geometric standard deviation) of the data will be completed following evaluation of the fit of the data to expected distributions (i.e., normal or log-normal) using standard methods for industrial hygiene data statistical analysis (Mulhausen and Damiano, 1998). If the data do not fit the two typically encountered distributions, either other distributions or Monte Carlo methods (Jayjock 1997,

Thompson, 1992) will be applied to analyze the data. Confidence intervals on the estimate of the arithmetic mean will also be determined. The extent of new monitoring to be conducted will depend on a) the extent of existing data, b) its applicability to the job(s) of interest, and c) the variability of exposures and the desired precision of current estimates of mean exposures. These aspects will not be known until the field data collection is underway.

Job-Exposure Matrix (JEM) and Individual Subject Exposure Matrix. Many occupational exposure assessments for epidemiology applications have applied a job-exposure matrix design (Gamble and Spirtas 1976). A series of standard facility types and job titles and time periods are developed. Then, based on historic monitoring data or retrospective estimation, exposures are provided for the cells in the matrix. Then, subjects' work histories are linked to the standard jobs and thus to exposure. JEMS have particular appeal in an industry where there are many workers who fit a relatively short list (compared to number of study subjects) of standard facilities and jobs. This JEM approach has been used for a major exposure assessment project in China (Dosemeci 1994), but the validity of those results has been critiqued (Budinsky 1999), with one of the issues being the rather broad industry and job categories used. For the disease progression study, subjects (cases, controls) will be recruited from Shanghai area hospitals, and there is no *a priori* reason to expect them to all arise from a narrow range of jobs and industries. Rather, there will likely be a broad group of industries and jobs, perhaps to the point of each subject representing a unique facility and/or job combination. Thus, for planning, the presumption is that each study subject will require his or her own unique exposure assessment. So, the efficiencies of a JEM with a standard set of jobs are unlikely to be widely applicable for characterizing the exposures of the exposed subjects. New exposure monitoring data will be collected for the subject, if returned to the same job as before study recruitment. Otherwise, monitoring of other workers in the same job(s) previously held by the subject will be used to estimate the subject's exposure. Monitoring of multiple workers will be undertaken as a means of evaluating the inter-worker variability (Rappaport, 1993).

Sampling to Define Long-Term Average (LTA) Exposure. Expected variability plays a large role in the number of samples needed to estimate an arithmetic mean to a desired level of certainty. The arithmetic mean is the preferred estimate for a long-term average exposure (Rappaport 1991). An individual exposure measurement from one day is not a reliable estimate of a worker's weekly, monthly, or annual average exposure (Hewett 1995). If we have limited data for an operation but enough so that we can estimate the geometric standard deviation (and not reject log-normality), then we can estimate the number of samples needed to define the arithmetic mean to a desired level of accuracy. However, substantial relaxing of usual criteria may be needed in order to set an achievable survey size. Resources available for monitoring may impact feasibility of meeting rigorous statistical design criteria. With this potential constraint in mind, the following provides an analysis of sampling requirements for different levels of certainty. Hewett (1995) provided the following estimates (Table 25.1). The range arises in part from uncertainty of the assigned GSD.

Table 25.1. Number of Survey Samples to Define the Arithmetic Mean (AM)
For an Exposure Group

	Target Accuracy for AM		
GSD	+/- 20%	+/- 30%	+/- 50%

1.5	18 to 34	8 to 15	3 to 6
2	59 to 119	28 to 53	10 to 19
3	225 to 452	100 to 201	38 to 72
4	589 to 1124	261 to 500	94 to 180

Jobs are comprised of one or more tasks. The job exposure variability arises from a number of causes, including variability in each component task's exposure. Much has been written on the topic of worker exposure variability (Mulhasuen and Damiano 1988, Rappaport, 1991, 1993), and work has been reported on statistical models of task-based exposures (Nicas, 1993 a and b). Much of the job variability statistical analysis extends to individual task variability analysis. It is largely only the averaging time that differs. For a whole job, the full shift is considered. For a task, it is the task duration that is relevant. Task exposures may have less variability than for the jobs (a composite of several task), so fewer measurements of each task exposure may be needed to adequately characterize the average and distribution.

23.8 Retrospective Exposure Assessment Approaches

To the extent historic exposure monitoring data are available, they will be the main basis for retrospective exposure assessments. However, in all probability, there will be jobs and industries for which the historic data are unacceptably sparse or are unavailable. This problem has been the topic of many publications in exposure assessment, and a number of resolution methods have been put forward. The main retrospective exposure assessment approach to be used for the disease progression study - deterministic modeling - is covered in more detail in Armstrong et al (1996) and others (Dodgson, 1987, Yu, 1990, Schneider, 1991). The methods maximizes the use of available exposure data in a comparative (rather than calculated from first principles) manner. We will also utilize a relatively new approach (Ramachandran 1999) that incorporates Bayesian methods to supplement a deterministic model. The Bayesian method facilitates the use of probability trees to characterize the uncertainties of information used as input variables for deterministic modeling of historic exposures. This treatment of probability distributions will also be an advantage for the pattern of exposure assessment.

The main steps in the retrospective assessment process are:

A. Assemble and analyze historic exposure measurements
B. Develop facility specific timelines of changes and determinants to describe those changes
C. Quantify the impact of each identified change determinant and extrapolate from available monitoring data
D. Fill in the subject's job-exposure matrix for each key time period/job in the work history
E. Identify gaps, assumptions and uncertainties. Complete recreations and monitor exposures where feasible and crucial to the estimating process.

These are discussed briefly below.

A Assemble historic exposure measurements.

The starting point will be the SMCDC database. From it, summaries of specific areas within covered factories will be completed. See Section 25.1 B for discussion of the initial data analysis. We will also seek prior records (before the database startup circa 1987) from the factories and summarize those data. Since methods have changed over time, we expect the need to demonstrate equivalence (or conversion factors) for any prior to current methods. We also expect a large number of area samples (instead of the preferable personal samples) and we will need to develop approaches to translate the area measurements (as feasible & as required) to better represent personal exposure estimates. The main approach will be a task-time weighted average approach (Smith 1991). The goal of the data assembly will be formation of a subject's job - exposure - time matrix.

B Develop facility timelines and history of key changes

We will develop and use standard questionnaires and data recording forms that are designed to assemble the history of key changes in the operations that could have altered exposure potential, generally in one of four categories:

Workplace/facility
Materials used
Environmental conditions
Tasks/work methods

Part of the effort will be identifying and investigating the key determinants of exposure. Within a given industry, there will be some consistency in these key determinants. Fortunately, operations have been rather stable in China until approximately the last decade. This relative stability should translate to simpler, fewer, and lesser magnitude changes to consider. Determinants of exposure are the factors that relate to the release, dispersal or contact with study chemicals leading to exposure of workers. Schneider (1991) discusses several "universal exposure modifiers." A few examples anticipated as applicable to Shanghai area industries are:

Workplace/Facility related
- process design - capacity, conditions, containment
- ventilation
- proximity to other contaminant sources

Materials related
- volumes of solvents used
- composition of solvents used

Environmental
- Climate - seasonal temperatures
- Local contaminant sources (other industry)

Tasks/work methods
- task duration and frequency
- work practices
- protective measures

C Quantify the Impact of the Changes and Extrapolate from Available Monitoring Data

The impact of a change will need to be established for each determinate. This will be somewhat on a case-by-case basis, although some determinants will be general and their quantitative impact can be generalized too. Several examples follow.

Let us presume we determine via a review of engineering drawings that ventilation capacity for a well-mixed factory room increased by twofold in 1985. By examination of a relevant indoor air concentration model (box model), we concentration is linear with respect to air volume. (Note other models, not all of which are linear, may be more relevant to other situations). This means doubling the exhaust volume would half the concentration in the room.
Current exposures (1986 to present) are 8 ppm
1985 and prior exposures = 8 ppm x 2 = 16 ppm

Let us presume we find records of laboratory analyses that show the benzene content of the solvent used is now 2% by volume (V/V), but prior to 1986 was 10% V/V. This represents a 5 x higher level in historic operations. Presuming volumes of solvent used, temperatures, etc. were equivalent, the prior to 1986 concentrations in air would be approximately 5 x higher. The actual ration of partial pressures relates to the mole fractions of benzene in the solvent, rather than the % V/V.

Additional *independent* determinants can also be considered. Correlated determinants can also be considered, but only if a mathematical function can be developed to account for their interaction. The discussion above illustrates the concepts with point estimates. However, where data are available to support the process, we will be treating the measured exposure data and the determinant based adjustments as probability distributions. The broad range of industries and jobs we expect to encounter will probably yield situations where documented information (for example, engineering drawings or solvent compositional analyses) is not always available or is uncertain. The Bayesian-deterministic model discussed above provides the framework to better incorporate uncertain information and probability in the retrospective estimating process. The calculations for incorporating probability distributions will employ Monte Carlo techniques.

D Fill In The Subject's Job-Exposure Matrix

Little need be said about this process. Simply, the results of the exposure assessment will be assembled with the subject's work history.

E Identify Gaps, Assumptions and Uncertainties. Complete Recreations and Monitor Exposures

Review of the work history and the exposure assessment results will be completed to identify gaps in the matrix. The review may show a missing segment where the jobs before and after are similar. Assuming similar exposure for the interval may bridge this gap, unless some information suggests an alternative approach is more appropriate, such as an indication that the subject was not employed for that segment. For situations where a job is suspected as involving benzene

exposure, but data and extrapolation are not possible, recreation may be considered. We do not expect recreation to be a routinely applied solution. To be useful, the recreation needs to accurately reflect the historic job situation, and doing so is no small undertaking. Also, the effort will possibly take significant labor. Thus, we will develop a list of jobs where recreation is a suitable approach, and prioritize them according the scope of impact on the assessment. For example, if several subjects have the same job in their work history, it would have a broader application than is just one subject is affected. From the list, we will determine if and for which recreations will be undertaken.

23.9 Exposure Assessment References

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24 DATA MANAGEMENT

Separate Clinical and Research databases will be maintained using FileMaker Pro. FileMaker Pro is a cross platform database program (works on both PC and Mac) that provides data input interfaces, creates and runs reports and exports for analysis. FileMaker Pro provides several levels of password protection for data input, data access, and data retrieval. Therefore, each participant will view only the data that is appropriate for their role in the study and prevent unauthorized access to protect patient/subject confidentiality and to maintain data integrity. The clinical database will be backed daily using Retrospect and an OnStream Echo drive.

24.1 Clinical Database

All patients will be entered into the clinical database when samples are received for diagnosis. Patient information, diagnosis, and test results will be entered and updated as necessary. This information will be made available to the attending physicians and patient confidentiality maintained according to standard clinical practice. The clinical database will be maintained in a secure manner within ICMRC and Fudan University IT with access limited to authorized laboratory personnel. At the end of the study, the clinical database will remain at the ICMRC at Fudan University Medical Center.

24.2 Research Databases

Data on subjects who meet the inclusion criteria for a given research study and who have given informed consent will accessible in the respective research databases. This will be done using relational database software methods so that there is no physical duplication of data files between the clinical and research databases, the integrity of the data will be maintained, but subject identities will not be accessible in the research databases. All study records will be maintained in a secure safe facility within ICMRC with access limited to authorized study personnel. In accordance with NIH-FDA/COMIRB guidelines, study records will be maintained for a period of at least three years after the close of the study. Four research databases will be maintained: AML/NHL case control study database, Disease Progression database, Molecular Epidemiology database and the Cell Culture database. This database will be preserved indefinitely as a resource for future studies.

24.3 Database Security

A Physical records

Hard copy records (consent forms, questionnaires, etc.) will be stored in locked cabinets at ICMRC. A secured file storage area has been constructed within the laboratory for this purpose Other laboratory data will be coded by study ID number and kept at ICMRC as previously described. Hard copy records will be maintained for three years after publication of final reports, and will remain in the sole custody of Fudan University during this time.

B Computer records

A layered protection scheme will be used to prevent unauthorized assess to study computers and data. The two means of data/computer access are from a remote location through the internet/LAN and direct assess using the computer that contains the data. Unauthorized internet/LAN access will be controlled using DoorStop firewall software. This program prevents access from any computer without an authorized IP address. Firewall monitoring software (Who's There?) will be used to determine if there are attempts being made to access the study computers. In addition, the operating system has user/password security features to prevent unauthorized access as well. Physical access to the computers will be limited to authorized personnel and user/passwords will be necessary in order to log onto the computers. In addition,

the individual FileMaker databases will be user/password protected as well. The combination of these security features will prevent unauthorized access to clinical and/or study data.

25 RECRUITMENT AND TRAINING OF LABORATORY STAFF

25.1 Recruitment

Laboratory staff were recruited and selected from qualified applicants in Shanghai and elsewhere in China. Our goal is to select the best applicants that meet qualifications acceptable by US guidelines (CAP, ASCP, FDA- see CAP Guidelines Feb21-2000). For example, the Chinese Laboratory Director should be a certified clinical pathologist or a PhD with appropriate training and/or clinical specialty certification in one or more aspects of laboratory operation, ie. cytogenetics, hematology, and 3-5 years of clinical and/or research laboratory experience. Other supervisory positions (e.g. deputy director, clinical hematologist, cytogeneticist etc.) should be staffed by individuals with comparable training and experience, ie. clinical pathologists, or individuals with a terminal degree (MD, PhD) in the same or related field (hematology, cytogenetics, molecular genetics etc.) and two or more years of postdoctoral laboratory experience. Technical positions will be recruited from the best applicants possible.

25.2 Training

Training of Shanghai laboratory personnel at UCHSC fulfilled two purposes. First, training of staff is necessary in order to meet study obligations to leave a fully staffed functioning clinical and molecular laboratory in Shanghai by the end of the study. Second, training and staffing of the laboratory is crucial in order to maintain a functional laboratory and fulfill study outcomes.

Cytogenetics has not yet become part of the routine cancer diagnostic work-up in China. Therefore, recruitment of Chinese personnel with appropriate training and experience in clinical cancer cytogenetics is expected to be difficult. In order to facilitate standardization of clinical cytogenetic analyses as outlined in this proposal, it was necessary to train Chinese personnel at UCHSC. The training was intended to prepare Shanghai personnel with the knowledge and skills to perform all cytogenetic preparations and analyses, to develop and implement quality assurance and quality control programs and to transfer the latest technologies and information to the Shanghai laboratory. UCHSC and Cincinnati Children's Hospital Medical Center personnel will provide expert consultation and training for their Chinese counterparts throughout the study.

II. Molecular Epidemiology of Benzene-Exposed Workers in Shanghai, China

II. MOLECULAR EPIDEMIOLOGY OF BENZENE-EXPOSED WORKERS IN SHANGHAI, CHINA

26 OBJECTIVES

The objective of this series of studies is to investigate the dose response relationship between benzene, peripheral blood abnormalities and the development of blood dyscrasias in Shanghai workers exposed to benzene and to characterize polymorphisms that permit identification of subjects at increased risk of developing benzene-induced toxicity.

26.1 Specific Aims

1. To define benzene dose response relationships for specific cytopenias, and to the extent possible, pancytopenia, benzene poisoning and various hematologic indices associated with bone marrow toxicity and leukemia development

2. To identify the most sensitive biomarkers of exposure (internal) in benzene-exposed individuals.

3. To define sources of variability between peripheral biomarkers of benzene exposure and internal biomarkers of exposusre and effect.

4. To identify genetic and phenotypic polymorphisms that are associated with susceptibility to benzene toxicity and determine whether these influence benzene dose-response, especially for specific cytopenias.

5. To ascertain the relationship between external benzene exposure and the delivered dose (peripheral blood concentration) of benzene metabolites.

27 BACKGROUND AND RATIONALE

27.1 Benzene Bone Marrow Toxicity and Leukemogenesis

It would be surprising if leukemia induction is the most sensitive outcome for benzene exposure, yet, this is the assumption upon which regulatory standards are based. This research is designed to evaluate earlier markers of effect that would be useful in setting occupational and environmental standards of exposure to benzene. Chronic exposure to high concentrations of benzene has long been associated with clinical evidence of bone marrow suppression. Early clinical reports together with experimental studies suggest that lymphocytopenia and thrombocytopenia may predominate in subacute benzene toxicity [1-7]. However, the earliest hematologic and molecular events associated with benzene bone marrow toxicity have never been characterized according to presently accepted diagnostic criteria, and even the question of

whether benzene exposure results in AA, MDS, or both cannot be directly confirmed from existing literature. Moreover, no meaningful dose-response data exists with which to determine the concentrations of benzene that result in hematologic or molecular changes in bone marrow cells in vivo, and the reliability and sensitivity of putative biomarkers of benzene exposure or effect are not known.

Prior to the conduct of this study, it was unknown whether or not benzene exposure was associated with the development of a distinct subtype of MDS. However, we have recently described a distinct form of dysplasia in workers previously exposed to benzene (BID). BID is associated with a distinct set of features suggestive of a role for T lymphocyte activation and chronic inflammatory response in the pathogenesis of the disease. Further, we have recently identified an association between the (-238A) polymorphism in the TNF-a gene, an inflammatory cytokine. In the future, it may be central to our understanding of the relationship between benzene exposure and the etiology of leukemia and related diseases that the relationship between chronic inflammation and the neoplastic process be defined.

27.2 Benzene Metabolism

Since the pioneering studies of R.T. Williams, D.V. Parke and associates over half a century ago, benzene metabolism and toxicity have been the subject of extensive study [27-36]. It is now generally accepted in the scientific and medical communities that benzene metabolism is a requirement for bone marrow toxicity, with the phenolic and hydroxy- metabolites of benzene featuring prominently in its toxicity. Moreover, a variety of in vivo and in vitro studies suggest a major role for the benzene metabolite, hydroquinone, and its terminal oxidation product, p-benzoquinone, in bone marrow toxicity and leukemogenesis [17;32;37-46]. Trans-,trans-muconic acid (tt-MA) is a ring opened metabolite of benzene that is found in urine of benzene- exposed animals and humans. From this it has been hypothesized that trans-,trans-muconaldehyde is a toxic intermediate in benzene metabolism [47]. However, to date there has been no direct demonstration of the presence of this molecule in any species in vivo.

Although phenol is the primary hydroxylation product of benzene, urinary phenol is not a reliable biomarker of exposure to benzene at concentrations below 5 ppm. This is due in part to its lack of specificity with multiple exogenous and endogenous sources, as well as rapid conjugation and conversion of phenol to other hydroxy- metabolites in vivo. Two other minor urinary metabolites of benzene that have been evaluated as biomarkers of benzene exposure are tt-MA and the conjugation product, S-phenylmercapturic acid (S-PMA). Recent comparisons of these two compounds as biomarkers of benzene exposure conclude that both S-PMA and tt-MA are sensitive biomarkers of benzene. However S-PMA is more sensitive and reliable in the sub-ppm range of benzene exposures [48;49]. Unfortunately, urinary tt-MA is subject to suppression in individuals concomitantly exposed to toluene and other substituted benzenes and can arise from dietary sorbic acid. Consequently, concomitant toluene exposure has been reported to pose a serious interference problem in using tt-MA to monitor benzene exposure [49]. Many of the benzene exposure venues we will encounter in this study also involve heavy exposure to substituted benzenes, such as toluene. Comparative evaluation of benzene metabolism in different exposure environments is not a specific aim in this study, and discrimination of benzene metabolic

pathways by analysis of urinary metabolites is not a feasible goal. Therefore, we have decided to forego analysis of tt-MA, and will utilize S-PMA and exhaled breath measurements as a monitor of benzene exposure.

27.3 Polymorphisms and Susceptibility to Benzene Toxicity

The role of genetic polymorphisms in conferring susceptibility to the development specific diseases and toxicity as a result of exposure to environmental agents is presently the subject of widespread interest. Genetic and phenotypic polymorphisms have been reported to coincide with increased susceptibility to benzene poisoning. Primary hydrolysis of benzene to phenol, hydroquinone and related hydroxy- metabolites in the liver is a requirement for toxicity, and the principal enzyme involved is cytochrome P-450 2E1 (CYP2E1) [33;35;36]. Hydroquinone and related hydroxy- metabolites are further oxidized in the bone marrow by myeloperoxidase (MPO) to benzoquinones which are thought to be the proximate toxic species [37;38;50]. The pattern of genetic polymorphisms in the human CYP 2E1 gene appears to be complex, subject to individual and ethnic variation, and it is unclear at present whether genetic polymorphisms affect expression or induction of CYP2E1 protein or enzyme activity [51]. CYP2E1 genetic polymorphisms have been reported not to impact the risk of BP in benzene-exposed workers; however, a phenotypic polymorphism in CYP2E1 hydroxylating activity has [50]. In light of the number and complexity of genetic polymorphisms at this locus, the analysis reported in the latter study cannot be considered to be definitive [50;51]. However, chlorzoxazone 6-hydroxylation has been demonstrated to selectively reflect CYP2E1 hydroxylating activity in vivo, and has been used to characterize a rapid and slow CYP2E1 phenotypic trait in a variety of populations [52-54]. The rapid hydroxylating phenotype has been reported to be associated with a 2.6-fold increased risk of BP [55]. Together, the combination of a rapid hydroxylating 2E1 phenotype and a homozygous mutation for the enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1) at base pair 609 has been reported to carry a 7.6-fold increased risk of BP compared to subjects who were slow hydroxylators and who carried one or two wild-type NQO1 genes [50]. Another evaluation of the potential association between P450 2D6 and 2E1 genotypes and risk of developing secondary or idiosyncratic AA proved negative [56].

NQO1 catalyzes the formation of hydroquinones from quinones without intermediate formation of semiquinone radicals [57]. Ross and coworkers first described a role for NQO1 in bone marrow stroma in detoxification of benzene in 1990 [58]. Subsequently, a homozygous mutation at position 609 in the NQO1 gene (C to T) has been described that leads to a lack of enzyme activity in vitro and in vivo (NQ01*2 allele) [59-61]. Moran et al have posited that this mutation results in the lack of enzyme induction in bone marrow stroma in response to oxidative stress [62]. Rothman and colleagues reported a 2.4 fold increased risk of BP in subjects exposed to benzene who were homozygous for the NQO1 609 mutation [55]. Alternatively, Larson and co-workers only observed a 1.4-1.6 fold risk of leukemia associated with the NQO1 609 polymorphism in patients receiving alkylating chemotherapy [63]. It is important to note that the NQ01*2 inactive allele is present at considerably higher frequency in Chinese populations (49%) than in Caucasians (16%) [64]. Independently, Naoe and colleagues have recently described a NQO1 polymorphism at codon 187 that is associated with an increased risk of treatment related AML (ser/ser) and an unexpected decreased risk of de novo AML (pro/ser) [65].

Glutathione S-transferase theta 1 (GSTT1) catalyzes the conjugation of foreign compounds with glutathione and is thus thought to play an important role in the enzymatic detoxification of environmental carcinogens. Polymorphisms at this locus have been associated with an increased risk of ovarian cancer of specific histologic subtypes [66]. At least seven independent studies have evaluated the influence of a homozygous deletion in GSTT1 on risk of developing MDS or AML [67]. While two studies have observed an increased relative risk of MDS or AML associated with this polymorphism, five have not. The explanations for this apparent discrepancy are not well understood but may include ethnic differences in the study populations, the potential toxicity of glutathionyl-S-quinone, and/or inability to resolve associations with individual AML subtypes or cytogenetic lesions. One potentially important observation is a relatively large disparity in the number of s-AML's, including those with –5 and –7 cytogenetic findings, that were available for comparison among these studies. Therefore, it is important to investigate the role of GSTT1 polymorphisms focusing primarily on the development of s-AML in combination with detailed cytogenetic analysis.

A deficiency in MPO is well described that involves an underlying missense mutation leading to truncation of the resulting protein product. This leads to a failure in proteolytic cleavage which is a necessary step in post-translational modification of the enzyme [68]. This deficiency in MPO is transmitted as a autosomal recessive and occurs at a frequency of about 0.05% in the general population [69;70]. This mutation would theoretically serve to protect against benzene bone marrow toxicity.

In addition to these polymorphisms, weaker arguments can be made to investigate an even larger array of potential susceptibility genes. Due to limitations of time and scope, we have decided only to directly evaluate NQ01, CYP2E1 and GSST1. However, a basic strategy employed in all three of these studies is to provide a library of DNA material that can be made available for further studies, should resources and adequate scientific justification allow. Because of the importance of confirming the quantitative association previously reported for NQO1 (genotype) and CYP2E1 (phenotype) and the relative risk of developing BP, we propose to directly examine these two polymorphisms in a subset of workers occupationally exposed to benzene and to correlate these findings with hematologic and cytogenetic measures of effect. Further, this study design is particularly well suited to addressing outstanding questions with respect to the role of the GSST1 genetic polymorphism in leukemia risk, particularly s-AML. However, because MPO activates benzene metabolites a deficiency would be expected to confer relative resistance to BP, an outcome which would not be readily detectable in this study. Independently, MPO deficiency is commonly encountered as an acquired trait in cases of MDS and AML, suggesting that the feasibility of assessing a role for MPO in BP in these studies is extremely low.

27.4 Bone Marrow Metabolism and Toxicity

An established literature has accumulated to characterize the quantitative effects of benzene metabolites on functional and cytogenetic outcomes in human bone marrow and lymphoid cells in vitro [17;25;42;44-46;71-79]. In many cases these events comport with prevailing theories on the pathogenesis of MDS and AML [45;80]. Nevertheless, extrapolation of these studies to humans has been limited because of the lack of direct validation of the relationship between tissue metabolite

concentrations and external benzene dose. Although benzene metabolism has been the subject of numerous investigations, no direct measurement of benzene metabolites in human bone marrow has ever been attempted following in vivo exposure to benzene.

In a cross-sectional study of occupationally exposed workers, Rothman et al conducted an indirect analysis of benzene metabolism by measuring the urinary excretion of phenol, catechol, hydroquinone and muconic acid [81]. Measuring the relative proportion of each metabolite excreted as a function of benzene exposure, these authors concluded that the risk of BP may be supralinear in relation to external dose, ie. that extrapolation of effects observed at high levels of exposure may underestimate the risks associated with lower levels of exposure. Interpretation of these results is hampered by the fact that mass balance was impossible to determine, and if a decrease in one metabolite might be offset by an increase in another unmeasured metabolite (or fate) then the results might, in part, be artifactual. Further, urinary metabolites do not necessarily reflect bone marrow concentrations [39]. Again, these questions would be resolved by direct information on the relationship between blood concentrations and the external dose of benzene.

27.5 Health Significance

Together with results obtained from the Disease Progression Study, data obtained from this study will provide a basis to answer the question of whether there is a practical threshold for the dose of benzene producing hematologic and molecular changes in bone marrow cells that are involved in the pathogenesis of AA, MDS or AML. Analysis of polymorphisms between benzene unexposed workers, benzene exposed workers with no bone marrow toxicity and benzene-exposed workers with bone marrow toxicity will serve to confirm and extend previous studies designed to identify susceptible populations. Finally, analysis of benzene metabolite concentrations in blood will link in vivo benzene exposure with previously characterized in vitro effects of benzene metabolites on hematopoietic and lymphoid target cell populations.

27.6 Experimental Design

A two-staged project in benzene-exposed workers is underway:

Phase 1: *Retrospective study of benzene and leukopenia.* (Addresses specific aim 1). This study is being conducted using existing clinical and exposure records. The study population (n = ~1000 workers) has been selected from Shanghai benzene-using facilities. Stratification of exposures is being attempted to approximate the following distribution: No benzene exposure< 0.5-1.0 ppm < 1.0-10.0 ppm < 10.0-40.0 ppm < 40 ppm and greater.

Phase 2a: *Cross sectional study of current benzene-exposed workers and hemotologic/cytogenetic outcomes.* The study population will overlap with the population identified for phase 1. This study is also being conducted in 5 facilities in which benzene exposure occurs. Peripheral blood is collected from each participant, and potential markers of effect determined. The study population consists of 500-750 workers. The goal is to identify a significant workers in each group that approximate the same distribution of exposures outlined in Phase 1. For a subset of these

workers, benzene metabolites are being measured in blood. Dose response relationships between both external and internal benzene exposure, and early markers of effect will be assessed (Specific Aim 2). The sources of variability and the relationship between internal and external exposure, as well as blood measures will also be assessed and compared to IH results and urinary biomarkers of benzene exposure (Specific Aim 3). Genetic and phenotypic polymorphisms will be assessed and compared in order to determine if these impact benzene dose response relationships (Specific Aim 4).

A Identification of the Study Populations and Enrollment of Subjects

A.1 Additional Inclusion Criteria

After the sites are selected, additional inclusion criteria will include: Male or female workers 18-67 years of age who have (a) worked at least one year in a station covered by existing monitoring data, (b) had at least one clinical examination recorded and available for use in the study, and (c) have no history of cancer, therapeutic radiation exposure, or chemotherapy, as indicated in the existing physical examination records.

A.2 Controls

In phase 1, the lowest dose group will serve as controls. Identical exclusion criteria are inherent for the lowest (control) and higher (exposed) dose groups. In phase 2, unexposed controls will be selected from one or more factory sites in Shanghai that have not used benzene or benzene containing solvents. Efforts will be made to exclude other potential toxic exposures (See Section 10). Identical inclusion criteria in terms of years worked will be employed in control selection, as well as identical exclusion criteria in terms of previous cancers, radiation and chemotherapy exposure.

B Analysis of Exposure

The criteria for including sites into the study is primarily based on complete, accurate records of retrospective exposure. Therefore initial records have been obtained to document facility exposure history. We will also examine site specific records, and will include sites with the best-documented benzene exposure history. Through previous site visits, we have examined site exposure records and have found them to be very well maintained. In the existing site records, benzene exposure concentrations are provided by job, station and site. Time-specific quantitative estimates of benzene exposure will be developed for each job/station/site combination. Inspection of the heterogeneity of exposure data by site may allow for estimating exposures for combined work settings.

B.1 Benzene Exposure Assessment

Phase 1 and 2. In the existing site records, benzene exposure concentrations are provided by job, station and site. Time-specific quantitative estimates of benzene exposure will be developed for each job/station/site combination. Inspection of the heterogeneity of exposure data by site may allow for estimating exposures for combined work settings.

For work settings in which previous benzene exposure is difficult to quantify or lacks appropriate data, supplemental workplace monitoring will be performed. The methods used to develop this additional monitoring data will be carefully compared to previous methods used for the existing exposure data. Comparable methods and/or appropriate adjustments will be made to preserve the comparability of previous and current benzene sampling data. Further specifications of the exposure assessment procedures are outlined in the Disease Progression Study.

For Phase 2a additional personal air sampling will be completed to expand the characterization of each subject's current exposure in order to form the basis for comparing the relationships between external exposure concentration, body benzene burden, and internal metabolite levels. In addition to the development of quantitative benzene exposure indices, analysis of exposure to toluene and xylenes is being evaluated and internal biological markers of benzene exposure are also being developed. These include phenol, hydroquinone and catechol in blood . Urinary biomarkers of benzene exposure are also being measured.

C Sampling Strategy for Health Outcomes

Phase 1 is a retrospective study that will assess levels of benzene exposure against periodically recorded white blood count data obtained from annual factory monitoring records. Facilities will be chosen based on past levels of benzene exposure. Every effort will be made to choose facilities so that the range of past benzene exposure is maximized.

Phase 2 is a cross-sectional study that will assess benzene exposure versus a variety of hematologic outcomes. Again, facilities will be chosen to maximize the range of exposure. However, since there is more emphasis on current, rather than past benzene exposure in phase 2, the facilities chosen may not be identical to those in phase 1. Parameters to be measured in Phase 2 include peripheral blood CBC. Parameters to be measured in Phase 2 include peripheral blood CBC, benzene metabolites, hydroquinone and catechol in blood and these metabolites and sPMA in urine. DNA is also being collected and genetic polymorphisms will be evaluated against outcomes and exposure.

D Potential Confounders and Effect Modifiers

Phase 1:

Information on potential confounders and effect modifiers are limited to items present in factory records of exposure and clinical examinations. For exposure, we will collect parallel

information on toluene and xylene exposure, which are documented in the factory records as "methyl benzene", and "dimethyl benzene". In addition, the following fields will be abstracted from clinical records and, pending relatively complete (i.e. unknown values <20%) information, assessed as potential confounders and/or effect modifiers in statistical models that assess exposure/white count relationships.

- occupational hazards
- preventive measures
- previous clinical history
- acute occupational history
- smoking
- alcohol use
- height/weight
- previous medication use
- current medications
- current diseases
- hobbies that may entail exposure
  pregnancy

Height and weight will be used to compute Quetelet's index [weight/height^2], which will be used as a surrogate measure of obesity.

Phase 2:

It is unlikely that countervailing workplace exposures will be potential confounders, since work environments with such exposures will be eliminated in the population selection step. If, however, there are small subgroups of workers with exposures that may have been missed upon study site selection, we will collect this information for all study subjects through workplace records and additional industrial hygiene (IH) sampling, if needed.

Factory records will be used to abstract information on:

- age
- start date
- previous jobs/exposures
  dates of first/last exposure

A questionnaire has been developed to assess potential confounders and effect modifiers in phase 2. The questionnaire will be administered by a trained interviewer who will not be aware of outcome measures.

28 BENEFITS

28.1 Contribution to the Worldwide Community

The proposed research will provide a detailed understanding of the dose response and pathogenesis of benzene-induced bone marrow injury and clarification of the earliest events occurring in and distinguishing characteristics of blood dyscrasias developing as a consequence of benzene exposure. This information can be used to enhance and refine efforts to protect human health. Exposure analysis will provide valuable information on the nature of the dose response for benzene-induced disease, and characterization of genetically determined susceptibility will provide information that will permit the future identification of individuals with potentially increased risk of benzene-induced disease and whether these individuals polymorphisms influence the dose response for benzene risks. Finally, understanding the quantitative relationship between the external dose of benzene and bone marrow concentrations of benzene metabolites will provide a link between mechanistic studies of benzene toxicity and leukemogenesis and in vivo exposure to benzene. Taken together, these studies will provide a strong scientific basis for evaluating the health effects of benzene exposure and improving regulatory decisions aimed at preventing health risks associated with benzene exposure.

28.2 Contribution to the Study Population Community

The Chinese National Committee on the Development of Occupational Standards has specifically requested that we conduct this study in support of their efforts to reduce the benzene occupational exposure standard in China. This research is designed to test for earlier markers of effect than leukemia that would be useful to setting occupational standards in China and elsewhere. These outcomes are required as a basis for occupational standard setting in China. Previous studies of benzene exposure, especially those conducted in China have not estimated exposure that is adequate for standard setting.

28.3 Contribution to Individual Members of the Study Population

JCML and study personnel will support and assist SMCDCP and Municipal Health Department personnel in their regulatory and investigative mission to monitor benzene exposure and assess health effects in benzene-exposed workers. All three groups will participate as full collaborators in this study. This will extend to identification of potential study sites, inspection of facilities and assessment of exposure and identification and recruitment of study subjects. The exposure and health assessment technologies employed by JCML will provide enhanced capabilities for evaluation of benzene exposure and the diagnosis of BP or adverse health effects in individual workers. Workers in this study identified with clinical conditions consistent with BP, according to Chinese law, will be identified, removed from a benzene-exposure environment, referred to participating hospitals and registered in the Municipal Health Department Database. Individuals identified with BP will be offered participation in the DP study including follow-up and review to confirm that the above actions have taken place. The clinical and molecular technologies JCML will employ will enable enhanced detection of BP and follow-up of individual workers than is currently available in China and will provide a direct benefit to these subjects in the detection and management of their disease.

29 SOCIAL IMPACTS AND ETHICAL IMPLICATIONS

29.1 Ethical Implications

The proposed study involves patient sampling and the use of biological specimens as well as the collection of personal data and genetic information. Therefore, the inclusion of human subjects will undergo ethical examination by an Ethical Committee convened by the Sponsors especially for this purpose, the Combined Multiple Institutional Review Board of the University of Colorado and Shanghai Municipal authorities. The study will also be conducted according to Good Laboratory Practice guidelines.

All study participants will be informed of the purpose of the study and will be asked as a group to sign an informed consent form. The results of these studies will be communicated to local community regulatory and medical authorities and the results of individual patient tests will be communicated to treating physicians. All study subjects will be compensated for participation in the study at levels reflecting the degree of inconvenience, the invasive procedures required and that are in keeping with local social and ethical standards of practice.

29.2 Patient/Subject Confidentiality

Subject confidentiality will be maintained at the level of analysis and dissemination of study data. The clinical laboratory activities in which JCML will be engaged require extremely high standards of reliability with respect to patient identification and communication of clinical data. Because it is impossible to blind laboratory personnel to patient identification, laboratory personnel will treat patient clinical information with appropriate professional standards of professional confidentiality. A separate study database will be maintained at UCHSC for dissemination and analysis of study data. The study database will be networked with the clinical database at JCML but will be access-protected using both password and firewall technology.

30 PROCEDURAL OVERVIEW AND REGULATORY COMPLIANCE

30.1 Summary of Study Procedures

During Phase 1, ICMRC and SMCDC personnel initially reviewed annual monitoring records, the BP database, and other information to select factories for evaluation on the basis of previous benzene exposure history. We will then assessed levels of benzene exposure against periodically recorded white blood count data obtained from annual factory monitoring records. The regular monitoring and reporting of hematologic outcome in uniform clinical records at potential study sites has been mandated by law for a number of years. Serial counts will be abstracted for different study subjects, along with the dates of WBC measurements. A secondary outcome measure for phase 1 will be hemoglobin (Hgb) which is also routinely reported in uniform clinical records. Serial measures and dates will also be abstracted for Hgb.

In Phase 2, a number of different sources have been used to identify potential study sites. In addition, a reasonable balance of gender and age groups will be sought, as in phase 1. All workers (exposed and controls) will be required to have worked in the facility at least one month. In addition, a reasonable percentage (20% or more) of longer-term workers (exposed for 10 or years or more) will be sought. Exclusion criteria will include a history of cancer, therapeutic radiation or chemotherapy exposure. Selected workers will be invited to participate in the study and informed consent obtained. Blood samples will be collected by venipuncture.

Findings considered to be clinically significant, and/or to indicate a diagnosis of BP is warranted will be communicated to 1) the SMCDCP and Municipal Health Bureau; 2) the patient's attending physician, and 3) the ME research database. Patients diagnosed with BP will be invited to participate in the Disease Progression Study and offered clinical follow-up. It is expected that some individuals will present with abnormal hematologic changes that are insufficient to warrant a diagnosis of BP. In such cases, we will communicate these findings to their treating physician.

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32 EPIDEMIOLOGIC PROCEDURES AND DETAILED STUDY DESIGN

32.1 Experimental Design

Phase 1 is a retrospective study that will assess levels of benzene exposure against periodically recorded white blood count data. Facilities will be chosen based on past levels of benzene exposure. Every effort will be made to choose facilities so that the range of past benzene exposure is maximized.

Phase 2 is a cross-sectional study that will assess benzene exposure versus a variety of hematologic and cytogenetic outcomes. Again, facilities will be chosen to maximize the range of exposure. However, since there is more emphasis on current, rather than past benzene exposure in phase 2, the facilities chosen may not be identical to those in phase 1.

32.2 Identification of the Study Population and Enrollment of Subjects

Phase 1: We have previously visited a number of facilities where benzene has been or is being used in Shanghai. These visits have allowed us to ascertain the feasibility of identifying worksites that have regularly recorded benzene concentrations for various workstations, as well as regularly recorded results of physical exams. The database of occupational exposures that is maintained by CDC will be used to facilitate the selection of study sites. To be considered a study site, there must be at least five years of recorded benzene exposure measurements, and five years of clinical examination results.

The final population selected will be targeted to have the following attributes:

Percent female: 50 – 80%

Age: 16-30: 30-60%, 30 – 45: 30 – 60%, 45+: 5 – 30%

In addition, the range of benzene exposures should be as great as possible in order to allow the study to detect effects. The benzene exposure data (by workstation) will be reviewed before selecting a site. Sites will be selected so that the final distribution of exposure (by workstations) is as follows:

Workstations: < 1 ppm: 10- 25%, 1 - 10 ppm: 20 – 35%, 10 – 40 ppm: 20 – 35%, > 40 ppm: 20 – 35%.

Selection of sites will be made with concurrence of plant management and workers or worker representatives.

After the sites are selected, additional inclusion criteria will include: (a) at least one year worked in a station covered by existing monitoring data, (b) at least one clinical examination recorded and available for use in the study, and (c) no history of cancer, therapeutic radiation exposure, or chemotherapy, as indicated in the existing physical examination records.

Phase 2: Phase 2 will recruit 800-1000 benzene-exposed and non-exposed control subjects. To the extent possible, the CDC occupational exposure database will again be used to identify potential study sites. However, sites that are not included in the database also have been selected. Sites will be chosen based on current benzene exposure.
The desired distribution of current exposure is as follows:

CONTROLS	<1-<10 PPM	10-<25PPM	25-<40PPM	40PPM+
200	200	200	200	200

In addition, a reasonable balance of gender and age groups will be sought, as in phase 1. All workers (exposed and controls) will be required to have worked in the facility at least one month. In addition, a reasonable percentage (20% or more) of longer-term workers (exposed for 10 or years or more) will be sought. Exclusion criteria will include a history of cancer, therapeutic radiation or chemotherapy exposure.

Phase 2. Selected workers will also be invited to participate in a more intensive study aimed at investigating the effect of benzene metabolism on health effects. The more highly exposed workers in the table above will be over-sampled.

32.3 Benzene Exposure Assessment

Phase 1 and 2: The criteria for including sites into the study is primarily based on complete, accurate records of retrospective exposure. The initial records that will be used to document a facility's exposure history will be the Shanghai CDC database on exposure. We will also examine site specific records, and will include sites with the best-documented benzene exposure history. Through previous site visits, we have examined site exposure records and have found them to be very well maintained.

In the existing site records, benzene exposure concentrations are provided by job, station and site. Time-specific quantitative estimates of benzene exposure will be developed for each job/station/site combination. Inspection of the heterogeneity of exposure data by site may allow for estimating exposures for combined work settings.

For worksettings in which previous benzene exposure is difficult to quantify or lacks appropriate data, supplemental workplace monitoring will be performed. The methods used to develop this additional monitoring data will be carefully compared to previous methods used for the existing exposure data. Comparable methods and/or appropriate adjustments will be made to preserve the comparability of previous and current benzene sampling data. Further specifications of the exposure assessment procedures are outlined in appendix ____.

Phase 2: In addition to the development of quantitative benzene exposure indices, internal biological markers of benzene exposure will be developed for Phase 2.

These include but are not limited to:

- benzene in blood
- hydroquinone in blood

Phase 2. In addition to peripheral blood measurements, blood hydroquinone concentrations will be used as a marker of internal dose if pilot studies prove the methodology is feasible. See Section 15 and Appendix N.

32.4 Assessment of Outcomes/Potential Health Effects

Phase 1. For phase 1, the outcome that will be assessed will be the total leukocyte (white blood cell) count in whole blood. This outcome is regularly reported in uniform clinical records at potential study sites for a number of years. Serial counts will be abstracted for different study subjects, along with the date of the white count. A secondary outcome measure for phase 1 will be the hemoglobin concentration (in grams per liter of whole blood), which is also routinely reported through uniform clinical records. Serial measures and dates will also be abstracted for hemoglobin concentrations. No other health outcomes possibly related to benzene exposure are recorded in existing clinical records.

Phase II will assess a number of other potential health outcomes and/or biologic marker of effect in study participants.

a) Preexisting/current disease(s). Any preexisting or current diseases related to benzene, namely aplastic anemia, benzene poisoning, and/or MDS/AML will be referred to the parallel disease progression study.
b) Hematologic measures. A 20 ml. sample of blood will be drawn at the end of the workshift for each study subject. Clinical measures will include a complete blood count, platelets, and absolute white counts, including lymphocytes.

32.5 Potential Confounders and Effect Modifiers

Phase 1:

Information on potential confounders and effect modifiers will be limited to items present in factory records of exposure and clinical examinations. For exposure, we will collect parallel information on toluene and xylene exposure, which are documented in the factory records as "methyl benzene", and "dimethyl benzene". In addition, the following fields will be abstracted from clinical records and, pending relatively complete (i.e. unknown values <20%) information, assessed as potential confounders and/or effect modifiers in statistical models that assess exposure/white count relationships.

- occupational hazards
- preventive measures
- previous clinical history

- acute occupational history
- smoking
- alcohol use
- height/weight

Height and weight will be used to compute Quetelet's index [weight/height^2], which will be used as a surrogate measure of obesity.

Phase 2:

It is unlikely that countervailing workplace exposures will be potential confounders, since work environments with such exposures will be eliminated in the population selection step. If, however, there are small subgroups of workers with exposures that may have been missed upon study site selection, we will collect this information for all study subjects through workplace records and additional IH sampling, if needed.

Factory records will be used to abstract information on:

- age
- start date
- previous jobs/exposures
- dates of first/last exposure

A questionnaire will be developed to assess other potential confounders and effect modifiers in phase 2. The questionnaire will be administered by a trained interviewer who will not be aware of outcome measures. The questionnaire will include:

- smoking history
- alcohol use
- previous medication use
- current medications
- previous disease history
- current diseases
- hobbies that may entail exposure
- pregnancy
- treating physician

The questionnaire will be pilot tested, and any necessary modifications will be made to assure valid responses.

33 STATISTICAL ANALYSIS AND INTERPRETATION

The overall strategy for epidemiologic analyses will be to (a) determine the effect of the independent variable (e.g. exposure) on the dependent variable (e.g. AML case, WBC, or cytogenetic effect) while accounting for effect modifiers and confounders, (b) determine the presence, influence and magnitude of effects due to effect modifiers (e.g. smoking, other exposures), (c) determine the presence, influence and magnitude of effects due to confounders (e.g. age, BMI), which can alter the effect between the independent and dependent variables.

For discrete variables, the statistical analyses will employ simple stratified analyses using Mantel-Haenszel models (Mantel and Haenszel, 1959) or logistic regression models (Breslow and Day, 1980,1987) as appropriate.

Statistical analyses will employ simple stratified analyses using Mantel-Haenszel statistics (Mantel and Haenszel, 1959), analysis of variance techniques and regression modeling. Both parametric and non-parametric techniques will be used, depending on required statistical distributional assumptions for parametric analyses. Statistical analyses will primarily be carried out in SAS, although other packages with common epidemiologic statistical routines (EGRET, EPICURE, STATA) will be used to supplement SAS analyses.

Continuous measures will be analyzed by appropriate linear model analyses (e.g. analysis of variance, regression, analysis of covariance [Snedecor and Cochran, 1989]). For the case of repeated or serial continuous measures, such as serial WBC, the analysis will be based on mixed-effect models appropriate for repeated measures on a single subject over time (Milliken and Johnson, 1984).

A special case for analysis is the role of genetic polymorphisms on exposure/response relationships that will be explored in subsequent analyses. For this assessment, each outcome will be characterized as a dichotomy and genetic polymorphisms, exposure etc. will be predictor variables (e.g. presence absence of chromosome 7q- deletions). In this manner, statistical analyses appropriate for case control studies can be employed as in Andrieu and Goldstein, (1998).

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34 EXPOSURE ASSESSMENT STRATEGY AND EXPOSURE MONITORING

34.1 Exposure Assessment Approaches

A Introduction

Many of the aspects and methods discussed in the Disease Progression (DP) Protocol, Section 27 Exposure Assessment apply to the Molecular Epidemiology (ME) studies. In this section, we will reference the DP where appropriate, discuss differences, and provide additional information relevant to the ME studies. See Figure 12.1 (following in Section 12.8) for an overview of the ME exposure assessment flow.

B Tier 1. Ordinal Range Exposure to Benzene

For the ME study, we will bypass the Tier 1 stage and begin at the Tier 2 exposure assessment stage from the DP discussion. However, aspects of study enrollment questionnaires, use of the SMCDCP database and other records will still be used to the extent possible to select study locations and personnel. By intent, we expect to limit the range of factory types and thus limit the range of potentially confounding co-exposures. However, a similar scheme to the DP Tier 1 will be used to assess exposures to any potential occupational exposure confounders.

C Tier 2. Quantitative Assessment

See Section 25 of the Disease Progression Study Protocol for additional information.

a. Air Monitoring

Individual job location exposure records exist, at least for factories evaluated in the study feasibility investigation. Relatively uncomplicated (and thus robust) job histories are expected as the norm. Steps in assembly of quantitative air exposure assessments include:

1) Work history initial development. For cases and controls, job information will be obtained from facility records. The SCDC database will then be queried for an initial profile of the factory and exposure potential. From the SCDC system, a classification into exposure categories will be completed.

2) Work history expansion. Where retrievable and when a full work history is needed, factory records and interviews will be used to expand the work history.

3) Exposure monitoring records. See the DP study discussion.

4) New monitoring. See the DP study discussion. However, for the Molecular Epidemiology Phase 2 studies, additional personal air sampling measurements will be obtained. The objective for is to characterize the extent and pattern of exposure for each job assignment and the extent and pattern of exposure for each subject at an individual level.

5) Reconstruction and monitoring. See the DP study discussion. However, for the ME study, we expect to base the initial retrospective study almost entirely on existing exposure records. Therefore, although reconstruction remains a possibility, its role is expected to be minimal.

b. Monitoring methods

See the DP study discussion of air monitoring methods.

2) Blood analytes. Measurements of benzene and co-exposures to aromatic hydrocarbons in blood will be performed. Analytes will include benzene, and other aromatic hydrocarbons as the parent compounds. Method: Benzene, toluene, xylenes by headspace [3] or gas-phase extraction, or equivalent methods.

D Tier 3. Assessing the Pattern of Exposure

See the DP study discussion.

c. Biological Monitoring

For Phase 2 of the molecular epidemiology study, various biological exposure monitoring techniques (as described in this section) will be applied. A variety of monitoring methodologies are be used to verify and supplement personal air sampling measurements for benzene, toluene, xylenes and other aromatic hydrocarbons. One of the research objectives for Phase 2 is to establish the relationships between external benzene exposures (e.g. air concentration via personal monitoring), external co-exposures to other aromatic hydrocarbons, blood metabolite concentrations.

Dermal contact potential will be evaluated qualitatively; its function will be to indicate whether of not significant dermal uptake occurred for a given subject so that external air to internal concentration relationships would be confounded. The goal of this dermal contact evaluation is not to quantitate the relative contributions from dermal versus inhalation routes. If dermal uptake potential is substantial, the exhaled breath biological monitoring data will give a measure of the total exposure, due to inhalation and dermal routes.

E Blinding

See the DP study discussion. However, for the ME study, since most of the aspects are prospective, blinding of the exposure assessment staff is not a significant factor.

34.2 Exposure Assessment Air Sampling and Analytical Procedures

See the DP study discussion.

34.3 Exposure Assessment - Direct Measurements with UltraRAE PID for Benzene

See the DP study discussion.

34.4 Exposure Assessment - Field Monitoring and Information Collection Procedures

See the DP study discussion.

34.5 Exposure Assessment - Quality Assurance Plan

See the DP study discussion.

34.6 Exposure Assessment - Exposure Monitoring Statistical Plan

See the DP study discussion. For the ME retrospective study and Phase 2, we expect to gain efficiencies via use of similar exposure groups [5] and determinations of exposure profiles for those similar exposure groups.

Exposure Assessment References

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35 BIOLOGICAL MONITORING

35.1 HEMATOLOGY

Hematology procedures are the same as those outlined in the Disease Progression Study (I).

36 ANALYSIS OF POLYMORPHISMS

36.1 Genetic Polymorphisms

Individual genetic polymorphisms will be analyzed in immortalized peripheral B lymphocytes as described in Section I.23 of the Disease Progression Study. Genetic polymorphisms to be evaluated in this study include: NQO1 and GSTT1. Additional potential susceptibility genes may be added at a later date depending on the availability of resources and subsequent development of the literature.

37 DATA MANAGEMENT

Separate Clinical and Research databases will be maintained as previously described. All reports and analyses will be based on aggregate data only. No identifiers, including study ID numbers, will be used in study reports. In addition every opportunity will be made to define certain variables (e.g. workplace) used in the report more generally so that a very specific combination of variables/traits cannot be used to identify individuals. These efforts will make it very unlikely that an individual could be identified through the final reports.

Hard copy records (consent forms, questionnaires, etc.) will be stored in locked cabinets at IBS. Other laboratory data will be coded by study ID number and kept at ICMRC as previously

described. Hard copy records will be maintained for three years after publication of final reports, and will remain in the sole custody of Fudan during this time.

Post-Study Professional and Technical Support

Final data review, collection, collation, analysis and migration will continue into 2008 in support of study closure and submission of resulting publications.