FLUOROCARBONS AND HUMAN HEALTH: STUDIES IN AN OCCUPATIONAL COHORT

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ABSTRACT

Perfluorooctanoic acid (PFOA) has been reported to be a nongenotoxic hepatocarcinogen and reproductive hormonal toxin in rats. Although PFOA is the major component of total fluorine in humans, little information is available concerning human toxicities. The health effects of PFOA were assessed in two studies conducted in occupationally exposed workers. The associations between PFOA and reproductive hormones, hepatic enzymes, lipoproteins, hematology parameters, and leukocyte counts were studied in 115 male employees. Serum PFOA was positively associated with estradiol and negatively associated with free testosterone (TF) but was not significantly associated with luteinizing hormone. The negative association between TF and PFOA was stronger in older men. Thyroid stimulating hormone and PFOA were positively associated. PFOA and prolactin were positively associated in moderate drinkers. The effect of adiposity on serum glutamyl oxaloacetic and glutamyl pyruvic transaminase decreased as PFOA increased. The induction of gamma glutamyl transferase by alcohol was decreased as PFOA increased. The effect of alcohol on HDL was reduced as PFOA increased. A positive association between hemoglobin, mean cellular volume, and leukocyte counts with PFOA was observed. These results suggest that PFOA affects male reproductive hormones and that the liver is not a significant site of toxicity in humans at the PFOA levels observed in this study. However, PFOA appears to modify hepatic and immune responses to xenobiotics. A retrospective cohort mortality study of 2788 male and 749 females workers employed between 1947-1984 at a PFOA production plant was conducted. Overall, there were no significantly increased cause specific SMRs. Among men, ten years of employment in PFOA production was associated with a significant three fold increase in prostate cancer mortality compared to no employment in production. Given the small number of prostate cancer deaths and the natural history of the disease, the association between production work and prostate cancer must be viewed as hypothesis generating and should not be over interpreted. If the prostate cancer mortality excess is related to PFOA, the results of the two studies suggest that PFOA may increase prostate cancer mortality through endocrine alterations.

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1. INTRODUCTION

Fluorine was first isolated as an element in 1880 by Moisser [1]. Five years later he synthesized the first fluorocarbons through uncontrolled reactions of carbon with elemental fluorine. It was not until the late 1930s that the controlled synthesis of fluorocarbons became possible. In the 1940s, Frigidaire and DuPont developed chlorofluorocarbons, the first commercially available fluorocarbons, for use in refrigeration [1]. During the same period perfluorocarbons, a subclass of perfluorinated organic fluorocarbons with unique properties, were first synthesized to meet the special needs of the Manhattan project [2]. The electrochemical fluorination method for perfluorocarbon production made commercial production of perfluorocarbons possible and opened the door to widespread use of perfluorocarbons [3, 4].

Fluorocarbons are wide ranging in their structures and uses. Many commercial applications have been developed for chlorofluorocarbon compounds including refrigeration, degreasing, aerosol dispensing, polymerization, polymer foam blowing, drugs, and reactive intermediates or catalysts. Perfluorocarbons (PFCs) have extensive applications because of their unique physical and chemical properties. These applications include use as artificial blood substitutes, computer coolants, polymers such as teflon, surfactants, lubricants, foaming agents, ski waxes, and in an extensive specialty chemical industry which produces grease and oil repellent coatings for paper and cloth, polymers, insecticides, and a variety of consumer products. Perfluorocarbons are currently being tested as replacements for chlorofluorocarbons in industrial processes and products.

For many years fluorocarbons were generally thought to be nontoxic. Perfluorocarbons were considered to be particularly nontoxic because they were chemically and physically inert and showed low acute toxicity in animals [4]. Recent epidemiological and experimental studies have associated exposure to chlorofluorocarbons, a subclass of fluorocarbons previously classified as nontoxic, with direct and indirect adverse human health effects. Subsequently, researchers and regulators turned their attention to the study of other fluorocarbons. The discovery that one perfluorocarbon, perfluorooctanoic acid

(PFOA), was present in measurable quantities in residents of several U.S. cities [5-7], the recognition that some perfluorocarbons including PFOA have long half lives in the humans [8] and the observations that PFOA produced toxic effects in animals, including hepatotoxicity, endocrine toxicity, immunotoxicity, and carcinogenesis [9], has led to a re-evaluation of the toxic potential of perfluorocarbons, particularly PFOA, in humans.

Despite widespread exposure to perfluorocarbons, little is known about their effects on human health. It was apparent that additional studies designed to explore their physiologic effects and potential adverse health outcomes and conducted in an occupational cohort with high exposure to PFCs, were necessary. The 3M Chemolite Plant located in Cottage Grove, Minnesota is one of a few PFC production facilities in the world. Biological monitoring data from studies of the Chemolite workforce showed that employees have had high levels and long durations of exposure to PFOA [8, 10]. This occupational cohort provided the opportunity to study the effects of PFOA on humans. The specific goals and objectives of this study were:

GOAL 1) To quantify the human effects of perfluorooctanoic acid on the following physiologic parameters:

a) Hormones: free and bound testosterone, estradiol, lutenizing hormone, thyroid stimulating hormone, prolactin, and follicle stimulating hormone.

b) Serum lipids and lipoproteins: cholesterol, low density lipoprotein, high density lipoprotein, and triglycerides.

c) Hematologic parameters: hemoglobin, mean corpuscular volume, white blood cell count, polymorphonuclear leukocyte count, band count, lymphocyte count, monocyte count, platelet count, eosinophil count, and basophil count.

d) Hepatic enzymes: serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, gamma glutamyl transferase, and alkaline phosphatase.

OBJECTIVE 1: to conduct a cross-sectional study of production workers to estimate the relationships between total serum fluoride, a surrogate assay for perfluorooctanoic acid, and physiologic parameters.

GOAL 2)To quantify the mortality in an occupational cohort with long term exposure to perfluorooctanoic acid production.

OBJECTIVE 2: to conduct a retrospective cohort occupational study to assess the mortality experience of workers using expected mortality based on Minnesota mortality rates.

2. REVIEW OF THE LITERATURE

2.1 Introduction

The presence of small amounts of fluoride in human blood was recognized in 1856 [11]. More than 100 years later, Taves [5, 6] presented evidence that fluorine exists in two major forms in humans and animals; in a free ionic state and in a covalently bound organic state. Prior to this report, it was assumed that fluorine existed primarily as inorganic ionic fluoride in biological systems. Taves' observations have since been confirmed by several other investigators [12-16]. The discovery that organofluorine compounds constitute the majority of fluorine found in humans focused research on characterizing these undefined compounds. Guy identified a perfluorinated compound, perfluorooctanoic acid (PFOA), as a major constituent of the serum organic fluorine fraction [7, 17]. Perfluorooctanoic acid (PFOA) is the only organic fluorine compound to be identified in human serum [18]. The recognition of human and animal toxicities associated with perfluorochemicals [9, 19], has renewed interest in understanding the human health effects of perfluorocarbons (PFC), particularly PFOA.

2 Organic Fluorochemicals

Organic fluorochemicals, otherwise referred to as fluorocarbons, are compounds composed of fluorine, carbon and other elements such as oxygen, nitrogen and sulfur. Perfluorocarbons have structures analogous to hydrocarbons, except the hydrogens are exhaustively replaced by fluorine [20]. A limited number of organic fluorochemicals occur in nature [21-23], however no PFCs occur naturally [24, 25].

The first report of the synthesis of a fluorocarbon was published in 1890 when Moissan claimed to have purified carbon tetrafluoride. It is likely he isolated fluorographite, however [1]. Pure carbon tetrafluoride was not obtained until 1930 [26]. Work by Ruff and the Belgian chemist, Swarts, in the late 19th and early 20th centuries laid the foundation of organic fluoride chemistry. Midegly and Henne extended Swarts' work and reported the synthesis of dichlorodifluormethane,

Cl_2F_2, in 1930 [27]. This chlorofluorocarbon with the trade name Freon 12 is an inert, non-toxic refrigerant which was vastly superior to other refrigerants available in the 1930s. After commercial production of Freon 12 began in 1936, it rapidly became a major industrial chemical [2, 26]. A number of cholorfluoromethanes and chlorofluoroethanes have been produced on a commercial scale in many regions of the world. These chlorofluorocarbons have been used in large amounts as aerosol propellants and degreasers, in addition to their use as refrigerants. Currently, their production is being reduced as a result of their ozone depleting properties [28, 29].

In 1937, Simons and Block developed a method to produce laboratory quantities of perfluorocarbons, such as C_3F_8, C_4F_{10}, cycloC_5F_{10} and cycloC_6F_{12} [2, 3]. The analysis of these compounds led to the understanding that many of the structures of saturated hydrocarbons could be replicated in the form of perfluorocarbons. Research in the area of perfluorocarbons was stimulated by two developments. First, Plunkett discovered the polymer, polytetrafluoroethylene, or Teflon [1]. Second, the development of perfluorocarbon chemistry was stimulated by the U.S. effort to develop atomic weapons during World War II under the Manhattan Project. The ^{235}U isotope of uranium was required for the development of atomic bombs. One method of uranium isotope separation was gaseous diffusion. The only volatile uranium compound available for use in this diffusion process was uranium hexafluoride, UF_6, an extremely reactive gas. Materials were needed for use as coolants, lubricants, sealers and buffer gases in equipment exposed to this highly reactive gas [1, 2, 26]. Perfluorocarbons prepared by Simons were found to be inert to UF_6. This discovery led to a research effort directed toward understanding the properties of a variety of perfluorocarbons and developing commercial methods for preparation of perfluorocarbons. The development by Simons of the electrochemical fluorination (ECF) was a major milestone in the fluorochemical industry. Since World War II there has been much interest and work in this new branch of organic chemistry based on perfluorocarbons.

The use of Simons' ECF method has allowed the production of a wide variety of perfluorocarbons including perfluorinated alkanes, alkenes, ethers, esters, amides, sulfonamides and compounds with cyclic and ring structures [2]. The 'inert' perfluorocarbons are compounds made up of only carbon and fluorine. This class

of compounds ranges from carbon tetrafluoride to complex multiple ring structures such as perfluorodecalin. Perfluorinated surfactants include carboxylic acids, sulfonic acids, and their derivatives. These compounds form the basis of an extensive fluorochemical industry. A variety of perfluorinated polymers and elastomers exist. The most widely used are polytetrafluoroethylene and Kel-F, a elastomer of vinyldiene fluoride and hexafluoropropylene.

2.3 Physical Properties

Perfluorooctanoic acid is a straight chain eight carbon carboxylic acid with a molecular weight of 414.16. The melting point of POFA is 59-60°C. Its boiling point is 189°C at standard conditions [30]. Perfluorooctanoic acid is produced as a complex mixture of branched chain isomers. In practice, all eight carbon carboxylic acid isomers are refered to as PFOA. The ammonium salt of PFOA (APFOA) is the common industrially used form of PFOA. It is a white crystalline powder that easily becomes airborne and sublimes at 130°C.

Perfluorocarbons have unique chemical and physical properties [20, 26, 31, 32]. The importance of perfluorination in producing these properties cannot be overemphasized. Perfluorocarbons are not just another hydrocarbon-like molecule. Chemically, perfluorocarbons are remarkably inert. They are stable to boiling in strong acids and bases. Very few oxidizing or reducing agents react appreciably with perfluorocarbons. Perfluorocarbons that contain other organic molecules such as nitrogen, oxygen and sulfur will participate in reaction at the site of these molecules. For instance, perfluoroctanoyl sulfonic acid will react and form the sulfonamide derivative. The amide portion of this molecule can then be conjugated with many other organic compounds. The perfluorinated portion of these larger molecules remains non-reactive.

Perfluorocarbons are heat stable. They can be heated to greater than 250°C without breakdown. At high temperatures, greater than 400°C, some compounds will breakdown. For example, PTFE, breaks down to perfluoroisobutylene (PFIB), an extremely toxic gas [1]. Because most perfluorochemicals are heat stable they are used in high temperature applications.

The inert perfluorocarbons are excellent insulators. Polymers, such as PTFE, and inerts PFCs, such as perfluorohexane, are used in electrical applications because of their superior dielectric properties. Their heat stability and insulation properties make perfluorocarbon materials the insulators of choice [20].

Perfluorinated surfaces are the most non-wettable and non-adhesive surfaces known [20, 26]. Fluorochemical surfactants are some of the most potent surface active agents yet discovered [31]. Very low concentrations of fluorochemical surfactants effectively reduce the surface tension at interphase boundaries.

Most perfluorocarbons are poorly soluble in both aqueous and organic solutions. They form a group of fluorophilic compounds, however some perfluorocarbons with functional groups such as the salts of PFOA, are highly water soluble [31, 32]. Perfluorocarbon liquids dissolve oxygen avidly. This unique property is the basis for the use of perfluorocarbons as blood substitutes [33].

Perfluorinated carboxylic and sulfonic acids are some of the strongest organic acids known [31]. The pK_a of PFOA is 2.5 [34]. Thus, when in physiologic solutions, they exist in primarily anionic forms. The anionic forms have a strong propensity to form complex ion pairs* .

In the past, some investigators have assumed that the chemical and physical properties of many fluorocarbons is synonymous with lack of activity in biologic systems [35, 36]. However, abundant evidence exists that their chemical and physical inertness does not imply biologic inertness [19, 30, 37, 38].

2.4 Synthesis

Synthesis of fluorocarbons has been accomplished using four major methods; electrochemical fluorination (ECF), direct fluorination, teleomerization, and catalytic methods using high valence heavy metals. The ECF was developed by Simons in 1941 [3]. The Simons process is the oldest commercial technique and remains a commercial method to obtain many perfluorocarbons. A solution of

* personal communication from James Johnson, 3M Corporation

organic substrate is electrolyzed in anhydrous HF at a low voltage, high current, nickel anode. The products of these electrolysis cell reactions are largely perfluorinated. The spectrum of material produced by the ECF process is defined by the starting material. Commercial products from this process include perfluoroalkanes, perfluoroalkyl ethers, perfluoroalkenes, perfluoroalkyl esters, perfluorotrialkyl amines, perfluorocarboxylic acids and perfluorosulfonic acids [2]. Products of ECF often include a significant proportion of complex isomers and fragmentation products. For example, ECF production of PFOA from straight chain octanoic acid produces 30% complex branch chain isomers [39]. The mixture of products from each ECF run is unpredictably variable. These isomeric mixes are difficult to separate and purify [33]. Workers producing PFCs using ECF may be exposed to a complex mixture that changes composition over time.

Direct fluorination is another method used to produce perfluorocarbons. It is not subjected to the impurity problems associated with the ECF process. Direct fluorination reacts fluorine gas with hydrocarbon substrate. Because fluorine gas is extremely reactive, direct fluorination is a technically difficult process and has only recently been pilot tested for commercial production of fluorocarbons.

World production of fluorocarbons is limited to a handful of commercial plants. The 3M Corporation operates PFC production plants in Minnesota, Illinois, Alabama and Antwerp, Belgium. A plant in Italy owned by a Japanese and Italian consortium produces limited amount of fluorocarbons. Perfluorocarbons are also produced in Germany and have been produced, in the past, in the former Soviet Union.

2.5 Sources Of Organic Fluoride Exposure

Guy [17] presented possible candidates for the organic fluorine constituents of human blood based on observation made during the isolation of PFOA from serum. The organic fluorine was not likely to be a macromolecule such as a protein or nucleic acid, because of its solubility in organic solvents such as ether or chloroform/methanol. It was not covalently bound to albumin since it was removed on charcoal at pH 3 at room temperature. The solubility characteristics suggested that multiple compounds existed with different polarities. The major

compound was a polar lipid like molecule that was identified as PFOA. Other less polar compounds appeared to be present. This data suggests that fluorocompounds other than PFOA were bound to albumin. These compounds were not esters of C_{13-18} fatty acids and were less polar than PFOA. Perfluorooctanyl sulfonamide (PFOS) and its derivative compounds fit this description and may be constituents of the organic fluorine fraction. Although exposure is probably low, the properties of PFOS suggest that it may accumulate to measurable levels.

In contrast to ionic fluoride, little has been reported concerning the organic fluorine content of water and beverages. The fluorine content of ground water is essentially all in ionic form. Some fluorochemicals, such as the perfluorinated carboxylic acid surfactants and their salts, are soluble in water. Such water soluble compounds may locally contaminate surface and ground water near industrial plants that use these compounds. Other perfluorinated compounds such as the alkanes, alkenes, and ethers are fluorophilic and are insoluble in aqueous solutions. Although data on the oral organic fluorine intake is limited, it is unlikely that water and beverages are significant sources of organic fluorine in humans.

The diet as a source of the organic fluorine found in human serum has been the subject of speculation [5, 6, 18, 40]. Non-perfluorinated fluorocompounds have found in biological systems. Marais showed that fluoroacetate was the compound responsible for toxicity from the poisonous plant *Dichapetalum cymosum* [41]. Other investigators have found plant species that synthesize fluoroacetate, fluorocitrate, and monofluorinated fatty acids. Peters reported that a few toxic plants produce fluoroacetate [42]. Fluoroacetate and fluorocitrate have been found in beans grown in high fluoride soil [23]. Peters [21] and Lovelace et al. [22] have reported the occurrence of fluorocitrate in a few plants and foods. In animals, the metabolic activation of fluoroacetate into (-)-erythro-fluorocitrate blocks the transport of citrate into the mitochondria and citrate breakdown by aconitase [42, 43]. Other omega-fatty acids with even numbers of carbon atoms are highly toxic as a result of oxidation that produces fluoroacetate. Fluorocitrate also undergoes rapid defluorination in rat liver in the presence of glutathione (GSH) [44]. Given the low environmental levels, the infrequent occurrence, the toxicity, and the rapid

metabolism of these compounds in mammalian species, it is unlikely that these monofluorinated compounds contribute substantially to the organic fluorine content in humans.

Taves measured the organic and inorganic fluorine in 93 food items [45]. No significant organic fluorine was found in the tested foods. Ophaug and Singer tested a market basket of food. They concluded that there was no significant organic fluorine content in food. Although food and beverages generally do not contain PFCs, it is possible that they may be contaminated by fluorochemical packaging materials. Water and grease repellent coatings in packaging material could leach into food items in small quantities. This could occur when materials that are not designed for microwave use are used in microwave ovens. Studies have not been reported that quantify human exposures from food packaging sources.

Perfluorocarbons are contained in many consumer products. Fluorocarbon surfactants such as PFOA, PFOS, and it's derivatives are present in window cleaning products, floor waxes and polishes, fabric and leather coatings and carpet and upholstery treatments [20]. Additionally these compounds are used to coat food wraps and are incorporated into plastic food storage bags. Fluorocarbons are the basis for a new generation of cross country ski waxes. Teflon and Teflon related products are widely used as lubricants, electrical insulators, heat and chemical stable gaskets and linings and in non-stick cookware. Fluoroalkanes such as perfluorohexane are being evaluated as CFC replacements. If perfluorohexane or other fluorocarbons are used as replacements for CFC's, consumer exposure from aerosols and other products will increase dramatically. PFC's have several experimental medical uses including use as blood substitutes, x-ray and magnetic resonance imaging contrast agents [46], vitreous replacement and in liquid ventilation therapeutic methods [47]. Recently, a potent fluorocarbon insecticide has been marketed to control fire ants [48].

Perfluorocarbons have a variety of industrial uses. Teflon and other polymers are used where heat stable and chemically inert liners, gaskets and lubricants are necessary. In addition, they are used as electrical insulators both in solid and

liquid form and used as inert non-conductive liquid coolants in electrical devices such as Cray supercomputers. Perfluorinated surfactants are important fire suppression materials. Perfluorocarbons have been used to control the metal vapors in electroplating processes and to prevent the release of toxic gases\ from landfills[20]. Perfluorocarbons are being considered to replace CFC's in many processes such as refrigeration, polymer foam blowing and building insulation. New applications are being continually developed for these unique compounds, making increased exposure to workers probable.

2.6 Toxicokinetics of PFOA

Since Taves and Guy's observations, perfluorocarboxylic acids, perfluorosulfonic acids and their derivatives have been the subject of numerous toxicokinetic and toxicodynamic studies in animals. These studies have focused primarily on two compounds, PFOA, and perfluorodecanoic acid (PFDA).

Perfluorooctanoic acid or its salts are well absorbed by ingestion, inhalation or dermal exposure. Absorption has been studied primarily in rats, although a number of other species have been studied.

Five male and five female rats were exposed to airborne APFOA for one hour. In this experiment the nominal air concentration of ammonium perfluorooctanoate was 18.6 mg/l. No animals died during the inhalation exposure or the 14 day post exposure observation period. Pooled serum samples contained 42 ppm of organic fluorine for males and 2 ppm for females. Inorganic fluoride content was 0.02 ppm for males and 0.01 ppm for females [9]. Kennedy and Hall [38] studied the inhalation toxicity in male rats of ammonium perfluoroctonate using both single dose and repeated dose schedules. They found a LC_{50} of 980 mg/m^3 for a 4 hour exposure placing PFOA in the moderately toxic by inhalation category. Following ten repeated doses at levels of 1.0, 7.6, and 84 mg/m^3 blood ammonium PFOA levels were obtained. At the 1.0 mg/m^3 level PFOA levels were 13 ppm, at the 7.6 mg/m^3 level PFOA levels were 47 ppm and at 84 mg/m3 level PFOA levels were 108 ppm. Therefore it appears that PFOA is well absorbed by inhalation. It should be noted that the exposures were to APFOA dust, the likely form for occupational exposure.

Ammonium perfluorooctonoate in food and PFOA administered by gavage in propylene glycol or corn oil vehicles are well absorbed in rats. In an acute oral LD_{50} study [9], rats displayed a dose dependent spectrum of toxicities indicating that PFOA was absorbed after ingestion. PFOA levels were not measured in this study. In a subacute oral toxicity study, rats were fed PFOA for 90 days [9]. Serum concentration of organic fluorine showed a dose response relationship in both sexes. A marked gender difference in organic fluorine levels was observed. Males had organic fluorine 50 times higher than females at each dose level.

Studies have since demonstrated excellent oral absorption of PFOA in a variety of species including rats, mice, guinea pigs, dogs, hamsters and monkeys [9, 19]. Of most immediate relevance to humans have been studies in a small number of rhesus monkeys [9]. In a 90 day oral toxicity study, monkeys were given 3, 10, and 30 mg/kg/day doses of APFOA. In monkeys at the 3 mg/kg/day dose, mean serum PFOA was 50 ppm in males and 58 ppm in females. At the same dose, males had 3 ppm and females 7 ppm in liver samples. At 10 mg/kg/day doses, male monkeys had a mean serum PFOA of 63 ppm and females 75 ppm. Liver levels were 9 and 10 ppm for males and females, respectively. Because all but 1 monkey died at the 30 and 100 mg/kg/day dose levels, only 1 serum sample from a male monkey in the 30 mg/kg/day dose group was available. In this monkey the serum level of PFOA was 145 ppm. In the 30 and 100 mg/kg/day dose group mean liver levels were greater than 100 ppm. Thus, the oral route of absorption may be a significant contributor to the body burden of PFOA in exposed workers.

Dermal absorption of PFOA has been studied in rats and rabbits. Ammonium perfluorooctanoate is a fine white powder that may come into contact with skin and be absorbed. In rats dermally exposed to ammonium perfluoroctonate at 4 dose levels, PFOA was absorbed in a dose dependent fashion [37]. In single dose dermal exposure experiments using rabbits, PFOA appeared to be absorbed. Levels of fluorine were not measured, but dose dependent toxic changes were noted [9]. In a multi-dose experiment, ten male and ten female rabbits were injected dermally with a 100 mg/kg dose of PFOA on a five day a week schedule for two weeks. Total serum fluorine levels were increased in a dose-dependent fashion. Dose-dependent changes in weight were noted [49]. From these studies,

it appears that dermal exposure to the salts of PFOA are absorbed in animals. In the past, Chemolite workers have been exposed to large dermal doses of ammonium perfluoroctonate. It appears that dermal exposure may have played a significant role in the absorption of PFOA in these workers. Upon recognition that PFOA could be absorbed dermally, work practices were changed and engineering controls were adopted that reduced dermal exposures. The role that dermal exposures currently play in PFOA absorption at Chemolite has not been well studied.

Once absorbed, PFOA enters the plasma probably by diffusing as a neutral ion pair. In plasma, PFOA is strongly bound to proteins in the serum with more than 97.5 percent in bound form [50]. It is likely that albumin is the major site for high affinity binding [5-7, 50-54]. There does not appear to be a sex difference in protein binding [50, 54]. Hanhijarvi et al. have suggested that protein binding is saturable in rats [55]. Using human serum, Ophaug and Singer [39] found that PFOA was 99% protein bound at PFOA levels up to 16 ppm total fluorine, however. Guy suggested that perfluorocarboxylic acids bind to albumin in a similar fashion to fatty acids [24]. This hypothesis is consistent with the results of several studies. Taves observed that the organic fraction of serum co-migrated with albumin during electrophoresis [6]. Dialysis and ultrafiltration studies observed the retention of organic fluorine during dialysis and ultrafiltration [7, 17, 56]. Belisle and Hagen reported that PFOA appeared to be strongly protein bound in human serum [51]. Extraction of PFOA from acidified water is quantitatively complete using hexane. When PFOA is extracted from plasma, recovery is only 35 percent. Plasma appeared to complex PFOA and PFDA. The partitioning of the bound into organic phase during extraction was more difficult and necessitated the use of more polar solvents. Klevens [53] suggested that CF_2 and CF_3 groups complex with polar groups that are present in the amino acids in proteins such as albumin. In protein precipitation studies using bovine serum albumin, PFOA bound to albumin at an estimated 28 binding sites per molecule [52]. Nordby and Luck studied the precipitation of human albumin by PFOA. Under acidic pH conditions, PFOA produced reversible precipitation of albumin [57] by binding to high affinity sites. These studies do not rule out significant binding to other plasma proteins or erythrocyte components. In studies using serum protein electrophoresis, the protein bound organic fluorine was distributed in a diffuse

pattern [6, 17] suggesting that PFOA protein binding may be nonspecific. The large amount bound to albumin may reflect the abundance of albumin in plasma and serum.

In rats, PFOA is distributed to all tissues studied except adipose tissue. The highest concentrations of PFOA are in the serum, liver, and kidneys. Ylinen et al. [34] studied the disposition of PFOA in male and female rats after single and 28 day oral dosing. After a single dose of 50 mg/kg, PFOA was concentrated in the serum. Twelve hours after dosing 40% of the PFOA dose was found in the serum of males and 10% in females. Males retained 3.5% of the dose in serum after 14 days. PFOA was retained in the liver for much longer than in serum. In females, the half-life of PFOA in liver was 60 hours compared to 24 hours in serum. In males the half-life was 210 hours in liver and 105 hours in serum. It is noteworthy that PFOA was not found in adipose tissue in detectable quantities. After 28 days of PFOA treatment, PFOA was distributed to the following sites in decending amounts: serum, liver, lung, spleen, brain, and testis. Again, no PFOA was found in adipose tissue. The distribution of PFO from serum to the tissues occurred in a dose dependent manner for females. In male rats, the concentrations of PFOA in testis and spleen followed a dose dependent trend. The levels in male rat serum and liver was the same for the 10 mg/kg and 30 mg/kg dose group. Johnson and Gibson [58, 59] studied the distribution of ^{14}C labeled ammonium perfluorooctonoate after a single iv dose in rats. Their findings were similar to those of Ylinen et al. The primary sites of distribution were the liver, kidneys, and plasma. Other sites, including adipose tissue, had less than 1% of the administered dose. The level of PFOA in the testis of male rats was not reported. As discussed previously, the 90 day oral toxicity study in rhesus monkeys showed that the relative amounts of PFOA in serum and liver was different in monkeys compared to rats. In the low dose group of monkeys (3 and 10 mg/kg/day) serum had 5 to 10 times the PFOA levels found in liver. However, at higher dose levels, the PFOA levels were equally distributed. Additionally, no sex differences were noted in the monkeys liver and serum PFOA levels.

There is no evidence that perfluorinated compounds including PFOA are biotransformed by living organisms. Several studies have examined whether

PFOA is conjugated or incorporated into tissue constituents such as triglycerides or lipids. Ylinen et al. found no evidence in Wistar rats for metabolism or incorporation of PFOA into lipids [34]. Although the lipid content in PFOA treated rats was different than that in untreated rats, Pastoor et al. did not find evidence for PFOA incorporation into lipids or of metabolism [60] Vander Heuval et al. showed that PFOA was not incorporated into triacylglycerols, phospholipids, or cholesterol esters in the liver, kidney, heart, fat pat, or testis of male or female rats [61]. No evidence has been found that PFOA is conjugated in phase II metabolism [61]. Kusilkis et al. studied the formation of activated coenzyme A (CoA) derivatives of PFOA using rat liver microsomes. They found no evidence for the formation of a CoA derivative.

Sex related differences in the toxicokinetics of PFOA have been reported for rats. The mechanism of PFOA excretion appears to be species-dependent since these gender differences are not seen in mice, monkeys, rabbits, or dogs [9, 62]. The half-life of PFOA in female rats has been estimated to be less than one day [39], whereas the half-life of PFOA in males is five to seven days [34, 38]. It is of note that PFDA does not exhibit this gender difference [63]. It is hypothesized that the sex differences in sensitivity to the toxicities of PFOA are as a result of the slower excretion of PFOA in male rats compared to female rats. Investigators have reported that rats have an estrogen-dependent active renal excretion mechanism for PFOA which can be inhibited by probenecid [50, 54]. As noted previously, females have a much shorter half-life than male rats. The half-life in males can be reduced by castration or estrogen administration. It can be reduced to the female half-life by a combination of castration and estrogen treatment. Estrogen administration alone is almost as effective as the combination of castration and estradiol treatment in reducing the PFOA half-life. This treatment increased the renal excretion of PFOA in male rats to those observed in female rats. Other investigators have reported that the gender difference in half-life depends on a testosterone mediated increase in PFOA tissue binding [64]. This hypothesis is consistent with the gender difference in tissue half-life discussed previously [34]. Johnson has suggested that the primary method of excretion in intact males is via the hepatobiliary route [58, 59]. He reported that cholestyramine enhanced the fecal elimination of carbon 14 labeled PFOA in male rats. These data suggest there was biliary excretion with enterohepatic circulation of PFOA, particularly in

male rats. However, in a male worker with high serum PFOA levels who was treated with cholestyramine, little if any change in excretion of PFOA was noted. In this study PFOA was excreted slowly in the urine.

In humans, the half-life of PFOA appears to be extremely long and is not sex dependent. Ubel and Griffith [8] reported kinetic data for one highly exposed worker. At the time he was removed from exposure his serum organic fluorine was 66 ppm, 80 percent of which was PFOA. Over the next 18 months his organic fluorine level decreased to 39 ppm. Urinary excretion of PFOA fell from 387 micrograms/24 hours to 80 micrograms/24 hours. The decline in organic fluorine levels was consistent with two compartment kinetics, with a calculated half-life of 2 to 5 years. Additional unpublished biological monitoring data from three Chemolite workers is consistent with the 2 to 5 year half-life. In the Chemolite workforce, male and female workers employed in jobs with similar PFOA exposure have increased PFOA levels. Since men and women with similar exposures have similar levels, a large gender difference in PFOA toxicokinetics is unlikely. Therefore, the relevance of the rat data in assessing the effects of PFOA in humans is questionable.

2.7 Toxicodynamics of PFOA

2.7.1 Male Reproductive Toxicities

Both PFOA and PFDA have been found to produce significant toxicities in the reproductive systems of male rodents [19, 63, 65]. The testis has been reported as the target organ of toxicity for both PFOA and PFDA [19, 66]. Additional evidence exists suggesting that these compounds affect the function of the hypothalamic-pituitary-gonad axis (HPG) [19, 65].

Perfluorodecanoic acid, but not PFOA, has been shown to produce degenerative changes in rat seminiferous tubules that could progress to tubular necrosis. Van Rafelghem et al. reported that a single ip dose of 50 mg/kg of PFDA, produced degenerative changes in rat seminiferous tubules 8 days after injection [66]. Similar but lesser changes were noted in the seminiferous tubules of hamsters and guinea pigs treated in the same manner. They reported no such change in

treated mice. Bookstaff and Moore [65] did not observe similar changes in rats treated with 20-80 mg/kg of PFDA. They used a different strain of rats in their experiments which is less susceptible to testicular toxicants than those used by Van Rafelghem et al. Thus, the effects of perfluorocarboxylic acids on seminiferous tubules may be limited to a specific compound, PFDA, in a specific strain of rats. The effects observed by Van Rafelghem et al. in other species were not consistent and did not demonstrate a dose-response relationship. In monkeys treated orally with PFOA, no compound related histopathologic changes in the seminiferous tubules were noted [8].

In a two year rat feeding study, PFOA treated animals were observed to have increased numbers of Leydig cell tumors*. Male and female rats were fed PFOA containing diets resulting in a mean intake of 1.5 and 15 mg/kg/day. A statistically significant increase in Leydig cell adenomas of 0%, 7%, and 14% in the control, low dose, and high dose groups, respectively, was observed at the end of the two year study. The result was statistically significant as a result of the unexpectedly low number of adenomas in control animals. Historically, CD rats experience a lifetime mean Leydig cell incidence of 6.3 percent with a range of 2 to 12 percent. The high dose group incidence is outside the expected range and may represent a compound related effect. Although the evidence was not definitive, it suggested that PFOA may alter the histology as well as the function of Leydig cells in rats. Perfluorooctanoic acid was not mutagenic in the standard tests including the Ames assay using five species of *Salmonella typhimurium* and in *Saccharomyces cerevisiae* [9]. Mammalian cell transformation assays using C3H 10T 1/2 cells were also negative [67]. These data suggest that PFOA is not a genotoxic xenobiotic. The increase in Leydig cell tumors may be the result of an epigenetic mechanism.

The observation that rats fed PFOA for 2 years had an increased incidence of Leydig cell adenomas prompted researchers to examine the hormonal effects of PFOA in male rats [19]. Adult male CD rats were treated orally with PFOA in doses of 1 to 50 mg/kg. Serum estradiol levels were elevated in the rats treated with more than 10 mg/kg of PFOA. In the highest dose group estradiol was 2.7 times

* Report: 3M Riker Laboratories. Two Year Oral Toxicity/Carcinogenicity Study of FC143 in Rats #281CR0012,1983

greater than the estradiol levels in pair fed control group rats. Serum testosterone levels were significantly decreased in a dose dependent manner when compared with *ad libitum* feed control animals. No significant differences were observed between the high dose rats and their pair fed controls, however. No significant differences were noted in serum luteinizing hormone (LH) levels. Additionally, the accessory sex organ relative weights of the highest group were significantly less than those of their pair-fed controls.

In order to clarify the site of PFOA action, Cook [19] conducted a set of challenge experiments in PFOA treated rats. The results of these experiments demonstrate that the altered testosterone levels were PFOA related. Human chorionic gonadotropin (hCG) challenge can be used to identify abnormalities in the steriodogenic pathway. Human chorionic gonadotropin binds to the LH receptors on Leydig cells and stimulates sex steroid hormone synthesis [68]. Abnormalities in Leydig cell function can be detected by challenging Leydig cells with hCG and measuring steroid hormone production. Similarly, abnormalities in pituitary secretion of gonadotropins can be identified using a gonadotropin releasing hormone (GnRH) challenge that stimulates LH release [69]. Hypothalamic dysfunction can be identified using a naloxone challenge to stimulate GnRH release [70]. In rats treated with PFOA for 14 days at the same dose level as the initial experiment, the Leydig cell production of testosterone was significantly blunted after hCG challenge in the highest dose group compared to ad libitum fed controls. A small, non-significant blunting of the testosterone production in response to GnRH and naloxone was observed. Following GnRH and naloxone stimulation, LH levels were not significantly different in the treatment and control animals. The hCG challenge showed that the decrease in testosterone in PFOA treated rats resulted from altered steroidogenesis in the Leydig cell. The results from the GnRH and naloxone stimulation were not definitive. The results were compatible with an effect at the pituitary level as well as at the Leydig cell level. Cook et al. examined the site at which testosterone steroidogenesis was affected by PFOA. Progesterone, 17 alpha-hydroxyprogesterone and androstenedione were measured after hCG challenge. Progesterone and 17 alpha-hydroxyprogesterone were unaffected. Androstenedione levels were significantly decreased in PFOA treated rats compared to controls. Given that the conversion of 17 alpha-hydroxyprogesterone to androstenedione by C17/20 lyase is

necessary for testosterone synthesis, these results suggest that decreased testosterone is the result of a block in this conversion step. In hCG stimulated rat Leydig cells, the 17 alpha hydroxylase/C-17/20 lyase is inhibited by estradiol. Taken together, these data are consistent with the hypothesis that the elevated estradiol levels associated with PFOA treatment inhibit the C-17/20 lyase enzyme and thereby depress testosterone levels. Cook et al. suggested that the blunted response of LH to low testosterone may be mediated, in part, by elevated estradiol levels. A subtle hypothalamic or pituitary effect may also be present, however. The mechanism for the estradiol elevation was not studied.

Perfluorodecanoic acid alters reproductive hormones in male rats in a fashion similar to PFOA. In male rats treated with doses of PFDA ranging from 20 to 80 mg/kg, given as a single ip dose, PFDA decreased plasma androgen levels in a dose dependent fashion [65]. Both plasma testosterone and 5-alpha dihydrotestosterone were significantly reduced. Compared to *ad libitum* fed control rat values, mean plasma testosterone was decreased by 88 percent in PFDA treated animals and DHT was decreased by 82 percent. These changes were reflected in accessory sex organ weight and histology. The changes in accessory sex organs after PFDA administration were found to be reversed by testosterone replacement. The PFDA decrease in androgens was the result of decreased responsiveness of Leydig cells to LH. There was no evidence for altered metabolism of testosterone. Additionally, plasma LH concentrations did not increase appropriately in the face of low plasma testosterone concentrations. This suggests that PFDA may alter the normal feedback mechanisms of the HPG axis.

It is of interest to note that 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), which, like PFOA, is a nongenotoxic rat carcinogen, a peroxisome proliferators, and an inducer of P-450 system, has been shown to produce hormonal effects in male rats similar to those observed for PFOA and PFDA. Moore et al. [71] studied the effect of TCDD on steroidogenesis in rat Leydig cells. Exposure of cell to TCDD resulted in depression testosterone and 5-alpha-DHT concentrations without altering LH concentration or testosterone metabolism. Moore concluded that TCDD treatment inhibits the early phase of the synthetic pathway and the mobilization of cholesterol to cytochrome $P450_{scc}$. However, Moore et al.

observed decreased estradiol. TCDD has been shown to increase the estrogen mediated feedback inhibition of LH secretion [72] Additionally, in studies using MCF-7 breast tumor cells, the antiestrogenic effect of TCDD was mediated by alterations in the cytochrome P450 metabolism of estradiol [73]. The decreased testosterone in rats could be mediated by the effect of TCDD on Leydig cells directly, by alterations in testosterone metabolism, or through increased negative feedback at the pituitary or hypothalamic level. Recently, reports from occupational studies of TCDD exposed workers have associated TCDD exposure with hormonal alterations in human males. Egeland et al. [74] reported that men with high TCDD levels had significantly depressed serum testosterone levels. The changes in testosterone were not associated with altered LH values. Estradiol values were not reported. They concluded that dioxin has a similar effects in men and male rodents. The obvservations that PFOA, PFDA, and TCDD have overlapping spectrums of rodent toxicities suggests that peroxisome proliferators, inducers of the P-450 system and non-genotoxic carcinogens may also alter the hypothalamic -pituitary-gonad function in male animals.

2.7.2 Female Reproductive Toxicities

In the two year rat feeding study, female rats treated with PFOA were observed to have an increased number of mammary fibroadenomas compared to control animals. All mammary carcinomas occurred in control animals. Hyperplasia of the ovarian stroma was observed, but specific histopathological studies were not reported * . No information is available concerning the effect of PFOA and PFDA on HPG axis in women or female animals.

2.7.3 Thyroid Toxicities

Altered thyroid hormone dynamics have been observed in rats exposed to PFDA [75-78]. A single ip dose of PFDA in rats results in a rapid and persistent decrease in thyroxin (T4) and T_3 [78]. Gutshall reported that the decrease in thyroid hormones occurred as early as eight hours after treatment and persistent for at least 90 days [79]. These changes were associated with a hypothyroid-like state in

* Report: 3M Riker Laboratories. Two Year Oral Toxicity/Carcinogenicity Study of FC143 in Rats #281CR0012,1983.

the treated rats. The alterations in serum thyroid levels occurred at dose levels that did not produce a hypothyroid syndrome [78]. Animals with depressed T4 levels were found to be metabolically euthyroid [77]. Replacement of T4 resulted in normal food intake, but did not reverse the hypothyroid-like syndrome of hypothermia and bradycardia [76]. This suggests that PFDA has a marked effect on cellular metabolism that is independent of its effect on thyroid homeostasis. The low T4 was thought to be a result of two mechanisms. First, PFDA readily displaces T4 from albumin which results in increased metabolic turn over of the hormone. Second, the response of the hypothalamic-pituitary-thyroid (HPT) axis appeared to be depressed as assessed by thyrotropin releasing hormone simulation testing [75]. In these studies, the animals had increased levels of thyroid responsive hepatic enzyme activities suggesting that the PFDA treated rats were not functionally hypothyroid. The histological appearance of the thyroid glands were unremarkable, although treated rats had significantly lower thyroid weights. TSH levels were not studied. No similar studies are available for PFOA. PFOA has been noted to produce a transient weight loss in treated rats [30]. The hypothyroid-like syndrome observed in PFDA treated rats has not been studied in PFOA treated rats, however. Since the thyroid hormone effects of PFDA do not cause the hypothyroid-like state in rats, PFOA may alter the HPT axis without producing this syndrome.

2.7.4 Hepatic Toxicities

The primary site of PFOA toxicity in rodents is the liver. Peroxisome proliferation (PP), induction of enzymes involved in ß-oxidation of fatty acids, and induction of cytochrome P450 occur after a single PFOA dose. Marked hepatomegaly has been noted coincident to the PP and enzyme induction. Increased liver size was the result of a combination of both hypertrophy and hyperplasia. Cell hypertrophy predominated after an initial burst of cell proliferation. The initial hyperplasia is evidenced by large hepatocytes and markers of DNA synthesis [80]. Areas of increased necrosis in the periportal regions have been observed [81].

The relationship between hepatic enlargement, peroxisome proliferation, and increased ß-oxidation is unclear. Xenobiotic induced changes in one specific peroxisomal enzyme are not necessarily linked to changes in other peroxisomal

enzymes or hepatic enlargement [82]. Studies have suggested that xenobiotic induced hepatomegaly and PP may be related to the endocrine status of experimental animals or to oxidative stress [80, 83-86]. Adrenal and thyroid hormones may play a role in peroxisomal proliferation. [80, 85]. Studies of clofibrate, a PP, have shown that endocrine manipulation can modify its hepatic effects. In adrenalectomized and thryoidectomized rats, clofibrate-induced hepatomegaly was reduced compared to the effect in control rats [83, 84]. Conversely, in thyroidectomized or hypophysectomized rats, clofibrate induced peroxisomal ß-oxidation enzymes were increased compared to normal rats [83]. Thottassery et al. compared the PFOA-induced hepatomegaly in normal rats, adrenalectomized rats and adrenalectomized rats with cortisol replacement [80]. They found that hepatomegaly was cortisol dependent and was primarily a result of hepatocyte hypertrophy. Hyperplastic responses were also cortisol dependent and were noted in periportal regions of the liver. Peroxisomal proliferation did not depend on cortisol and was observed in centrilobular regions. They concluded that PFOA-induced hepatomegaly and peroxisome proliferation were separate processes.

In oral feeding studies, PFOA and other PP were reported to cause increased hepatomegaly in males compared to females. This difference could be reduced by exogenous estradiol administration or castration and eliminated by castration and estradiol administration [54]. These observations may be explained by an estrogen dependent renal excretion mechanism or a testosterone mediated increase in tissue binding [85, 87, 88]

Issemann and Green have cloned a mouse PP activated receptor, mPPAR, a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors that is activated by peroxisome proliferators [89]. This receptor directly mediates the effects of peroxisome proliferators (PPs). Tugwood has shown that PPs activated PPAR recognizes a specific response unit on the Acyl-CoA oxidase gene promoter in a manner similar to the steroid hormone receptor [90]. The action of PFOA and other PPs may be mediated by a family of cytosolic receptors that regulate gene transcription in a manner similar to other nuclear hormone receptors.

2.7.5 Nongenotoxic Carcinogenesis

In initiation, selection, and promotion experiments in rats, PFOA produced an increased number of hepatocellular carcinomas [91, 92] Several mechanisms for PFOA associated nongenotoxic carcinogenesis have been suggested.

Perfluorooctanoic acid is an archetypal member of a unique sub class of PPs that are not metabolized. Reddy has argued that the structurally diverse peroxisome proliferators (PP) are a distinct class of nongenotoxic carcinogens [86]. Reddy proposed that PPs induce oxidative stress which results in increased tumor formation. According to this theory, the observed increase in hydrogen peroxide formation associated with increased ß-oxidation is not associated with an increase of similar magnitude in detoxifying catalase activity [86]. Oxidative attack by hydrogen peroxide and other reactive oxygen species on cell constituents and membranes leads to DNA damage and increased cell proliferation. Increased proliferation in concert with DNA damage produces increased cell transformation and malignancies.

Studies testing the theory that PFOA induces HCC by increasing oxidative stress have lead to conflicting results. Takagi et al. observed an increase in 8-hydroxydeoxyguanosine in liver DNA from rats exposed to PFOA. They concluded that rat hepatocytes were under increased oxidative stress [93]. Handler et al. found no increase in hydrogen peroxide production in intact livers exposed to PFOA [94]. Lake et al. failed to find an association between hepatic tumor formation and peroxisome proliferation [95]. Thottassery et al. observed that the PFOA induction of ß-oxidation was independent of adrenal hormone status. A PFOA associated increase in catalase activity depended on cortisol [80]. Therefore, the hormonal status in animals used in experiments could confound studies of oxidative stress and account for the conflicting results.

2.7.6 Immunotoxicity

In the 90 day monkey feeding study, bone marrow and lymphoid tissue were a site of histopathology [9]. Treated monkeys in the highest two dose groups were observed to have moderate hypocellularity of the bone marrow. Specific

histopathological findings were not reported. Atrophy of lymphoid follicles in lymph nodes and the spleen were noted in the same treatment groups. No follow-up studies of these observations have been reported. Studies in PFOA treated rats have not shown histological changes in the immune system [9].

2.7.7 Mechanisms of Action

The mechanism of toxicity of perfluorinated surfactants may be mediated by their effect on cell membranes. Olson and Andersen [30] suggested that PFOA may alter membrane function through changes in fatty acid composition and oxidation status. Levitt and Liss hypothesized that the effect of perfluorinated surfactants is mediated by their alteration of membrane organization or fluidity [96, 97].

Shindo [32] reported that miscibility of fluorocarbon and hydrocarbon surfactants depends strongly on carbon chain length. A carbon chain length greater than eight carbons is necessary for immiscibility. Perfluorocarbon surfactants with eight or fewer carbon atoms are miscible with hydrocarbon surfactants with carbon chain lengths up to nine. These observations could have important implications for biological systems that contain fluorocarbon surfactants. Cellular membranes are a phase boundary, usually between a lipid phase and an aqueous phase. Surfactants will segregate to this phase boundary. Two immiscible surfactants may form two coexistent monolayers on the inside and outside of the membrane whereas miscible surfactants will form only one such monolayer. The presence of two monolayers will maximally reduce the surface tension at the boundary, whereas a single monolayer will affect surface tension to a lesser degree. Changes in surface tension may alter membrane fluidity and affect its function in such processes as signal recognition and transduction. It is interesting to note that the change in miscibility in Shindo's experimental system occurred for fluorocarbon surfactants with carbon chain lengths greater than eight. This change in miscibility depended on hydrocarbon surfactant chain length as well.

The effects of PFOA and PFDA on experimental membrane systems and cellular membranes have been investigated. Inoue studied the differential effects of octanoic acid and perfluorooctanoic acid on experimental cell membrane

properties [98]. The phase transition temperature of dipalmitoylphosphatidylcholine vesicles decreased linearly as PFOA increased in concentration up to one mM and then reach a plateau. This suggested that PFOA may form aggregates in the membrane above a critical concentration. Such a phase separation is observed to occur in micelles [32]. The partition coefficient between water and the membranes for PFOA, K = 8910, was larger than the coefficient for ionized octanoic acid, K = 135, possibly because of the difference in hydrophobicity between hydrocarbon and fluorocarbon chains in aqueous solution. The differences between the toxicokinetics and toxicodynamics of PFOA and PFDA may be the result of their differing miscibilities with cell membrane surfactants.

Levitt and Liss investigated the effect of PFOA and PFDA on the plasma membranes of cells from F4 human B-lymphoblastoid cell line using the dye merocyanine 540 (MC540) [97]. The dye binds to phospholipids that are loosely packed on the outer cell membrane, but does not bind to highly organized lipids and does not penetrate the membrane of healthy cells [99]. A large decrease in MC540 cell surface binding was observed after treatment with sub-lethal concentrations of PFOA and PFDA but not other non-perfluorinated fatty acids. Albumin or serum reduced the change in MC540 binding. This effect may be a result of the strong protein binding of PFOA and PFDA by albumin [50]. These observations suggest that PFOA and PFDA either interact directly with MC540 lipid binding sites or alter the structure of the lipids in the membranes.

In experiments examining functional changes in the lymphoblastoid cell lines, Levitt and Liss observed that PFOA and PFDA could cause direct damage to cells resulting in the release of membrane bound cell proteins and immunoglobulins in soluble form [96]. PFDA was significantly more potent than PFOA in solubilizing proteins and killing cells. This may be the result of different miscibilities in the cell membrane of these compounds. However, neither PFOA nor PFDA reduces the ability of surface immunoglobulins to migrate and undergo capping after antigen recognition [97]. In the PFOA concentration ranges that decreased MC540 binding, PFOA did not affect immunoglobulin migration and capping. Capping involves the cytoskeletal mediated polar migration of immunoglobulins within the plane of the membrane [100]. Apparently, the PFOA

and PFDA associated membrane changes do not affect membrane characteristics that are important for receptor migration.

The membrane effects of PFDA have been studied in greater detail. Pilcher et al. reported that a single injection of PFDA in rats significantly reduced the apparent number of β adrenergic receptors in cardiac cells [101]. This change in number of receptors was reflected in the diminished response of adenylyl cyclase (AC) to epinephrine in PFDA treated rat cardiac cells. The intrinsic properties of AC were not altered. The action of PFOA was on the epinephrine receptor. The fatty acid composition of the treated rat cardiac cell membranes was significantly altered [101]. Palmitic (16:0) acid was elevated 13 percent, eicosotrienoic (20:3 w6) was elevated 71 percent, and docosahexaenoic acid (22:6 w3) was elevated 18 percent. Arachidonic acid (20:4) was reduced by 18 percent. Several other investigators have reported changes in membrane function following PFDA exposure. Wigler and Shaw [102] demonstrated that PFDA inactivated a membrane transport channel for 2-aminopurine in L 5178 Y mouse lymphoma cells. *In vitro* experiments reported by Olson et al. [103] showed that erythrocytes exposed to PFDA exhibited decreased osmotic fragility and increased fluidity. Taken together, these studies indicate that perfluorinated surfactants exert their effects on cell membranes. The effects appear to be limited to the outer portion of the membranes as the result of differential partitioning within the membrane or binding to specific membrane constituents. Although PFOA and PFDA can be cytotoxic as a result of their detergent action on membranes, their membrane effects at lower doses are not related to their detergent action. From available data, it appears that functional membrane changes may be limited to specific receptor mediated functions.

2.8 Occupational Fluorine Exposures At Chemolite

In workers employed in fluorochemical production plants, blood organic fluorine has far outweighed ionic fluoride [8, 12, 14, 51, 56]. More than 98 percent of the total fluorine in these groups has been reported to be organic fluorine. Therefore, the use of total fluoride levels, which consist predominantly of organic fluorine compounds, is a valid surrogate for organic fluorine in occupationally exposed groups. In workers at the Chemolite plant, PFOA has been identified in the serum

of these workers and was estimated to account for 90 percent of organic fluorine found in the serum samples [8]. In this cohort of workers, total fluorine is a good surrogate measure for PFOA.

Industrial hygiene measurement of fluorochemicals have been conducted at the Chemolite plant since the 1970s [8]. These measurements include area samples, personal breathing samples and surface wipe samples. In 1977, a comprehensive effort at evaluating fluorochemical exposures was conducted at the Chemolite plant. During certain operations breathing zone PFOA concentrations were as high as 165 ppm. After extensive engineering control alterations, the plant was serially re-surveyed. In general, airborne exposures were below the recommended limit of 0.1mg/m3. However, there was evidence of surface contamination in production buildings [8]. In 1986, airborne PFOA, as well as breathing zone samples were less than 0.1 mg/m^3 based on 8 hour time weighted averages. Levels as high as 1.5 mg/m^3 were measured in breathing zone samples during certain clean-up and maintenance zone samples. Perfluorobutyric acid was also found, but in much lower concentrations. Spray dryer operators had consistently higher exposures, even following extensive equipment improvements. *

It appears that airborne exposure to PFOA was low for most workers. Spray dry operators and workers involved in clean up and maintenance activities have higher intermittent exposures. Although personal protection devices are required in high exposure jobs, worker compliance has not been evaluated. The role that surface contamination plays in worker exposure has not been defined* . The route of PFOA exposure in worker has not been clearly identified.

2.9 Epidemiological Studies

A retrospective cohort mortality study of employees at the Chemolite Plant in the period of 1948-1978 was conducted by Mandel and Schuman [8]. Of the 3,688 male employees who were employed for at least 6 months, 159 deaths were identified. There was no excess mortality in the employees as compared to all

* personal communication from Stan Sorenson, 3M Corporate Medical Department
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cause or cause specific mortality in the U.S. white male population. The subcohort of all chemical division workers did not show any all cause or cause-specific excess in mortality.

Starting in 1976 medical surveillance examinations were offered to Chemolite employees in the Chemical division [8]. Approximately 90 percent of the workers participated in the program. No health problems related to the exposure to fluorocarbons were encountered in participants. Serially conducted surveillance examinations have failed to reveal any relationship between blood levels of organic fluorine and clinical pathology *.

2.10 Summary

Animal studies have suggested that there are five areas of toxicity associated with PFOA exposure. These include hepatotoxicity, immune system alterations, reproductive hormone alterations, Leydig cell adenomas, and non-genotoxic hepatocarcinogenicity. Toxicity studies have primarily used rodents. There is considerable variability between strains of rats for some of the toxic endpoints such as Leydig cell adenomas. Additionally, some of the effects seen in rats have not been seen in other rodent species such as mice, hamsters or guinea pigs. The limited data available on PFOA exposed rhesus monkeys and occupationally exposed workers suggests that any extrapolation of the results from rodent experiments to humans requires more information about the mechanism of PFOA toxicity. From this data it does not appear that the liver is a major site for PFOA toxicity in humans. Of greater human health concern are the potential effects on the immune system and the reproductive hormones.

In the past, workers have been found to have significant blood levels of PFOA. Many workers have levels above one ppm. These blood levels are 50-1000 times background levels in the general population. These levels may be high enough to produce toxicities in occupationally exposed humans. A confident estimate of risk cannot be made until further information on the adverse health effects of PFOA exposure in humans is obtained.

* personal communication from Larry Zobel; 3M Corporation Medical Department

3. METHODS

3.1 Introduction

The effects of perfluorooctanoic acid (PFOA) exposure on human health were studied in employees of the 3M Chemolite plant (hereafter referred to as Chemolite) located in Cottage Grove, Minnesota. Two studies were conducted to investigate of the human health effects associated with PFOA exposure. First, mortality associated with occupational PFOA exposure was studied using a retrospective cohort design. Second, a cross sectional study design was used to estimate the relationships between PFOA exposure and selected physiologic parameters.

A retrospective cohort study was designed to examine mortality among workers. All workers ever employed at the Chemolite plant for greater than six months were included in the cohort. All causes and cause-specific mortality were compared to expected mortality. Expected mortality was calculated by applying sex and race specific quinquennial age, calendar period, and cause-specific mortality rates for the United States and Minnesota populations to the distribution of observed person-time [104, 105]. Age adjusted standardized rate ratios were calculated [106]. A relative risk (RR) for PFOA exposed workers compared to unexposed workers was calculated using proportional hazard regression models [107]. The RR were stratified by gender and adjusted for age at first employment, duration of employment and calendar period of first employment. Any significant differences between observed and expected cause-specific mortality were to be explored using nested case control studies. Case studies were completed for causes of death with 5 or more deaths and standardized mortality rates greater than 1.5. Each deceased individual's record was examined for commonalties in job history information including age at first employment, calendar period of employment, years in the Chemical Division, and duration of employment.

Selected physiologic effects of PFOA exposure were studied using a cross sectional study design. The relationships between total serum fluorine and biochemical parameters including reproductive hormones, hepatic biochemical parameters, lipid and lipoprotein parameters, and hematologic parameters, were

explored. A sample of the work force employed on November 1, 1990 was invited to participate. All employees in high exposure jobs were asked to participate. A sample of workers employed in low exposure jobs was frequency matched to the age and sex distribution of the high exposure group. Each participant completed a questionnaire which included medical history and information concerning alcohol, tobacco, and medication use. The questionnaire is provided in Appendix 3-1. Blood was drawn for determination of hematologic and biochemical parameters. Total serum fluorine, free (FT) and bound testosterone (BT), estradiol (E), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), prolactin (P) and luteinizing hormone (LH) were assayed. The PFOA-hormone dose-response relationship for each hormone was estimated using linear regression techniques to adjust for the effects of age, sex, body mass, alcohol consumption, tobacco use, and other potential confounders. The PFOA-hormone dose-response relationship was further explored by fitting linear multivariate models to hormone ratios. All unique ratios between the seven hormones were defined . Twenty-one hormone ratios were calculated for each participant. The prevalence of hormone values outside the laboratory reference range for men was compared to the expected prevalence assuming a normal distribution for assay values.

3.2 Retrospective Cohort Mortality Study

3.2.1 Definition Of The Cohort

The Chemolite facility opened in 1947. Individuals who were employed at the Chemolite plant between January 1, 1947 and December 31, 1983 were identified from company records. Workers with fewer than six months employment were excluded. In October 1951 large scale commercial PFOA production facilities became operational (Abe 1982). Because large scale PFOA production did not begin until 1951, a second cohort with potentially significant PFOA exposure was defined as those workers employed between October 1, 1951 and December 31, 1983. Subjects with greater than six months employment were included in this second PFOA cohort.

The cohort was initially assembled in 1979. Subsequently, the cohort was updated to include new employees through 1983. Personnel records for employees working prior to 1979 were coded for demographic items and work history by trained abstractors. Computerized corporate personnel databases were utilized to provide information for workers employed in the 1979 to 1983 period. Abstracted work history included year of first employment, year of last employment, years employed at Chemolite, and months worked in the chemical division. Individual job histories were not abstracted because job titles were defined by wage grades and did not correspond to specific jobs or locations within the plant.

3.2.2 Study Databases And Files

A Chemolite cohort database was created on a VAX computer using Ingres software. Data stored on magnetic tape were transferred to the VAX. Duplicate records were identified and removed. Missing data were identified. The Ingress update function was used for data editing. Final analytic files for the Monson program, SAS programs, and custom programs were constructed using the Ingress report writer.

3.2.3 Data Editing

The Ingres relational database allowed extensive internal consistency checks to be made. All dates were checked for plausibility. Those records with implausible, inconsistent, or improperly formatted dates were edited and corrected if information was available. Records of workers with fewer than six months employment were flagged and excluded from the analytic data set. A random check of 50 of the 364 workers with fewer than six month employment found no errors in classification of employment length. Extensive attempts were made to obtain all missing data items. Sources of information included plant personnel records, corporate personnel databases, benefit records, archived corporate records, plant medical records, and death certificates. No individual employees or next-of-kin were contacted. Four employees were excluded from the cohort as a result of missing demographic data items.

3.2.4 Validation Of The Historical Cohort Information

3.2.4.1 Assessment Of Completeness Of Ascertainment

The cohort was initially defined from personnel records stored at the Chemolite plant. Complete records were maintained on all workers ever employed at the plant. Hourly and salaried workers were included in these files, as were all transferred, terminated and retired former employees. Records for workers first employed in the 1947-1978 period were abstracted from documents, coded and computerized. A corporate computerized database was used to update the cohort through December 1, 1983. Since insufficient induction time had lapsed between 1983 and 1989, no new employees or work history information was added to the cohort database for the post 1983 period for this study.

Verifying the ascertainment of all eligible cohort members was problematic. The assumption that the personnel records represented a complete roster was difficult to check because of a lack of independent information. Several sources were used to exclude major errors in the enumeration of the cohort. The historical plant hiring pattern based on seniority dates was compared with the distribution of dates of first employment. Qualitatively, dates of major plant expansion corresponded to peaks in the distribution of dates of first employment and to seniority dates. Large increases in hiring due to new plant openings were reflected in peaks in the distribution of starting dates in the cohort. A sample of 25 annuity beneficiaries retired from the Chemolite plant were obtained from the corporate personnel office. All 25 were found to be included in the enumerated cohort.

Several plant personnel record systems were randomly sampled. Separate files were maintained for active workers, retirees, transferred and terminated workers, and workers whose employment at Chemolite ended prior to 1960. A sample of records for current employees with start dates prior to December 31, 1983 was compared to the cohort. All 12 records from the 1945-1960 period for start dates were found in the cohort database. Of 30 records sampled from the 1961-1969, 28 (93%) were included in the cohort. Fifty two records had starting dates in the 1970-1978 period. Of these 52 records, forty seven (90%) were found in the

database. In the 1979-1980 period 18 of 44 (41%) records were in the database. Lastly, in the 1981 through 1983 period, 36 of 37 records were in the database (97%). The low ascertainment for workers first employed in the 1979-1980 period was further examined. Of the 34 workers not in the cohort database, 16 (47%) were first employed in the 7/79-1/80 period. These omissions occurred in the transition period between document abstracting and electronic updating of the cohort. Using seniority lists, 44 workers currently employed were hired between 1979 and 1980. They represent approximately 1% of the total number of individuals in the workforce and less than 0.5% of the total person time at risk for the cohort. Records for retired workers were sampled from files containing all workers retired from Chemolite. Forty seven of the 48 (98%) sampled records were present in the database. A sample of the files containing the personnel records of employees completing employment before 1960 was randomly drawn. Of the 67 selected records, 65 (97%) were in the database. Finally, files containing records of all transferred, terminated, or disabled employees were randomly sampled. Of the 120 sampled records, 116 (97%) were present in the cohort database.

3.2.4.2 Validation Of Cohort Information

Information in the edited database was compared to information in the personnel records. A random sample of 25 records was drawn from the personnel files. Database names, social security numbers (SSN), dates of birth (DOB), and dates of employment were verified against record information. The sole error occurred in coding the last digit of one SSN. All other information was correctly entered into the database.

The reliability of ICD8 coding of death certificates for underlying cause of death was evaluated by resubmitting a sample of death certificates for coding by the same nosologist. The sample consisted of 25 death certificates from 1970 -1989. No change in the major categories of cause of death was noted. All cancer deaths were coded concordantly. Within cardiovascular causes of death, two certificates were discordant.

3.2.5 Vital Status Ascertainment

The vital status was ascertained from the Social Security Administration (SSA) and the National Death Index (NDI). All individuals with unknown vital status were traced successfully and vital status determined. Vital status determination in the 1979-1989 period was obtained through the NDI. Death certificates were requested from the appropriate state health departments for those individuals identified as, or presumed to be, deceased. A professional nosologist coded the death certificates for underlying cause of death according to International Classification of Diseases, 8th revision (ICD8). Information concerning the date and cause of two deaths which occurred outside the United States was obtained from family members or other available sources. Date of death and the ICD8 code for the underlying cause of death were entered into the database.

3.2.6 Validation of Vital Status Ascertainment

The vital status determination procedures for the cohort was evaluated. Corporate benefit records were utilized as an independent source for vital status among the retirees. Vital status from the database was compared to vital status in corporate records. A list of all retirees in the 1947-1984 cohort was sent to 3M benefits department. These individuals were matched to retirees who had received 3M death benefits. 3M records were not complete for periods prior to 1975. In the pre-1983 period, 4 deaths in retirees were identified by 3M records. Vital status was correctly ascertained by the SSA matching procedurefor only one of these retirees. In the 1983-1989 period, 34 deaths in retirees were identified in 3M records. The NDI matching procedure ascertained all 34 of these deaths. The NDI was not available for 1990. 3M records indicate that 8 retirees died during 1990. The incomplete SSA ascertainment in the period 1975 to 1983 resulted in extending the NDI search to include 1979 to 1983. All 3M identified deaths were also identified in the subsequent NDI search covering the 1979 to 1983 period.

3.2.7 Analysis

Analytic methods employed in this study were appropriate for cohort studies. The relative risk was estimated by calculating an adjusted standardized mortality ratio

(SMR) [105]. This study used both national and Minnesota mortality rates for comparisons. Mortality for men in the Chemolite cohort was compared to expected national and Minnesota mortality, adjusted for age, calendar period, sex and race. The use of mortality rates in the rural counties surrounding the plant were not considered to be stable for many causes of death and were not used. Since less than one percent of plant employees are non-white, white male and female rates were used for comparison. For women, only U.S. rates were used because cause- and calendar period-specific Minnesota rates were not available. SMRs were calculated for all cause, all cancer, and cause-specific mortality. The effects of disease latency, duration of employment, duration of follow-up, and work in the Chemical Division were examined using stratified SMR analyses.

Three additional methods of analysis were used to assess the validity of the SMR contrasts. The three methods were: standardized rate ratios (SRR) [106], Mantel Haenszel adjusted relative rates (RR_{MH}) [108], and proportional hazard regression adjusted RR [107].

Limited exposure data were available from plant records. Exposed workers were defined as all workers who worked for 1 month or more in the chemical division. Exposed and unexposed workers' all cause, all cancer, and cause-specific mortality was compared using stratified SMRs, SRRs [106], and stratified Mantel Haenszel analysis [108, 109]. Additionally, the same summary measures were calculated contrasting the rates for workers with at least ten years duration of employment and those with less than ten years employment.

The relative risk (RR) and 95% CI for the RR for deaths from all causes, cancer, cardiovascular diseases, and selected specific causes were estimated using a proportional hazard model (PH) [107, 109]. The time to event or censoring was defined as time from first employment to event or December 31, 1989. In PH models for specific causes of death, deaths from other causes were censored at the time of death. Exposure was quantified by months of chemical division employment. Covariates included in the models were age at first employment, year of first employment, and duration of employment. The analyses were stratified by gender. The appropriateness of the proportional hazard assumptions were tested using stratified models with graphical analysis of log (-log(survival))

versus follow-up time relationships and models that tested the significance of a product term between exposure and log(follow-up time) [109, 110].

3.3 Cross Sectional Study Of PFOA Exposed Workers

3.3.1 Population Definition And Recruitment

Medical screening of workers employed at the Chemolite plant occurs every two years. The general medical screening program included a medical questionnaire (Appendix 3-1), measurement of height, weight and vital signs, pulmonary function evaluation, urinalysis, serum assays, and hematology indices. This screening program offered an opportunity to assess the physiologic effects of PFOA exposure in workers engaged in commercial production of a limited spectrum of PFCs. Of particular interest were the effects of PFOA, the primary fluorochemical found in the serum of Chemolite workers. (Griffith and Ubel, 1980).

Participation in the Physiologic Effects Study required the subjects' willingness to undergo hormonal and biochemical testing and to have an additional 15 ml of blood drawn for total fluorine assay. In the cross-sectional study, exposure classification was based on the potential for PFOA exposure in a workers job and plant location. All workers engaged in any facet of PFOA production in the previous five years were considered to have potentially high PFOA exposure. The jobs considered to have high exposure potential included all jobs in the production buildings (bldg 6 and 15), all maintenance workers who were assigned to the PFOA production areas, and all management jobs requiring physical presence in the production building. Plant records and job history information was used to assign exposure status to individual workers. A random sample of workers in jobs with low exposure potential was frequency matched to the age and sex distribution of the high exposure workers. Workers with low exposure potential were defined as those assigned to jobs not involved in the production of PFCs for at least five years. A roster of workers meeting the low exposure potential was defined from plant records and knowledge of plant personnel about the location of high exposure jobs. A gender stratified sample from the group of workers in low exposure jobs with an age (5 year strata) distribution similar to the exposed group was identified and invited to participate. If a worker in a job with

low exposure declined to participate, another worker in the same age and sex stratum was randomly selected and invited to participate. In all cases informed consent was obtained. Participation in this study was voluntary.

3.3.2 Data Collection

3.3.2.1 Study Logs And Files

A roster of participants was maintained by the plant occupational health nurses. A log for biological sample information was completed by the laboratory technician. The date and time of ample coolection was recorded. Quality assurance samples were recorded on a separate log. Results reported on paper records were maintained as medical records. Results for other tests were transmitted electronically to a computerized database and coded as SAS datasets. All records were stored with employee medical records or in the corporate medical offices for confidentiality purposes. Printed laboratory results and questionnaire data were entered into a SAS dataset.

3.3.2.2 Questionnaire

Each participant completed a medical questionnaire prior to reporting to the plant medical office. (Appendix 3-1) Items included demographic information, symptoms, illness history and diagnoses, and medication usage. Detailed questions concerning tobacco use and alcohol use were included. Workers were not re-contacted to obtain missing information or to correct inconsistencies. Responses were not validated. Two plant occupational health nurses collected the questionnaires and returned them to the corporate medical department. In the corporate medical office, data were coded and entered into a SAS data base.

3.3.2.3 Laboratory Procedures

3.3.2.3.1 Height and Weight

Upon reporting to the plant medical office, participants had their height and weight determined by an occupational health nurse. Height and weight were measured once on the same calibrated scale.

3.3.2.3.2 Blood

3.3.2.3.2.1 Drawing And Handling

Four vacutainers of blood were drawn from a single venipuncture by a laboratory technologist. Two 15 ml red top vacutainers of blood were drawn and allowed to clot. One 10 ml purple top vacutainer was drawn for hematology studies. A specially prepared fluorine free 15 ml vacutainer was used to collect blood for total serum fluorine determination. Venipunctures were scheduled to occur at the same time of day and on the same shift for each worker. All blood was drawn between 6:30 and 8:00 a.m. Workers in the Chemical Division of the Chemolite plant rotate shifts on a weekly basis. Blood was drawn after a worker was assigned to the day shift for at least 3 days.

All specimens were refrigerated at the plant prior to transport to the appropriate laboratory. Clotted red top vacutainer specimens were centrifuged for 12 minutes to separate serum from cells before transport to the contract laboratory. In order to render the total serum fluorine specimens non-infectious, serum for total fluorine assays was ether extracted in the corporate medical department prior to sending the samples to the 3M Chemical Division analytic laboratories.

3.3.2.3.2.2 Assays

Serum samples were analyzed for total serum fluorine, hepatic biochemical parameters, cholesterol, lipoproteins, and seven hormones. Assayed biochemical parameters included serum glutamic oxaloacetic transaminase (SGOT), serum glutamatic pyruvic transaminase (SGPT), gamma glutamyl transferase (GGT), and alkaline phospatase (AKPH). The following hormones were assayed: bound testosterone, free testosterone, estradiol, prolactin, luteinizing hormone (LH), follicle stimulating hormone(FSH), and thyroid stimulating hormone (TSH). EDTA preserved whole blood samples underwent routine hematologic analysis including

complete blood count with erythrocyte indices and leukocyte differential cell count (CBC). Analyses were done without knowledge of the subject status or purpose of the study.

Total serum fluorine was determined in 3M's Chemical Division analytic laboratory using the sodium biphenyl extraction method (Venkateswarlu, 1982). The accurate determination of total fluorine in the parts per million (ppm) range required specialized equipment, procedures, and personnel. Assays were completed in a dedicated laboratory following tested protocols.

Upon receipt of extracted serum samples divided aliquots were frozen at -70 degrees centigrade. After all samples had been received, batches of 15 samples were assayed on successive working days. Each batch included high and low quality control samples. Each sample was assayed twice. If the difference in assayed values was greater than 1 ppm, the sample was re-assayed. The total serum fluorine value was reported as a mean value and a rounded integer value.

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamatic pyruvic transaminase (SGPT), gamma glutamyl transferase (GGT), and alkaline phospatase (AKPH) were assayed by the United Health Services Laboratory in Apple Valley, Minnesota using clinical colorimetric assays. CBCs were determined using automated Coulter counters. Light microscopy was utilized for differential counts.

Estradiol, prolactin, thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicle stimulating hormone (FSH) were assayed by the United Health Services laboratory using radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). FSH, LH, and prolactin were assayed using Abbott laboratories IMX microparticle enzyme linked immunoassays. TSH was assayed using London Diagnostics chemiluminescense immunometric assay. Estradiol was determined using Diagnostic Products Corporation's Coat-a-count assay.

Testosterone was assayed by the Mayo Clinic clinical laboratories. Total testosterone was determined by RIA using proprietary immunoglobulins. Free and bound testosterone was determined using equilibrium dialysis.[111].

3.3.2.3.2.3 Quality Assurance

Two methods were used to assess the accuracy and reliability of the laboratory assays. The laboratories routinely followed quality assurance programs. Three standards were run with each batch. If the control values were outside two standard deviations of the intra assay mean value for each standard, the assay was repeated. If 10 controls were outside 1 standard deviation of the mean, the assay was flagged for review. The reliability of each of these assays was assessed. For each assay, five specimens were randomly selected and split into two aliquots. The aliquots were labeled with different identifiers ensuring that the assays were carried out in a blinded fashion. Both aliquots were submitted on the same day to the laboratory. The coefficient of variation was calculated for each hormone.

3.3.3 Analysis

There were two analytic strategies. First, assay results were treated as continuous parameters and modeled using regression methods. Models were fit to assess the relationship between assay results and total fluorine, body mass index, alcohol consumption, and smoking. Second, hormonal assay results were dichotomized into those within the reference range and those outside the reference range. The hormone assay categories were based on published sex specific normal reference values for each assay. The purpose of this dichotomization was to evaluate the possibility that highly susceptible individuals may be affected at lower levels of exposure and not follow the adjusted dose-response curve.

The relationships between total serum fluorine and the assayed parameters were estimated by fitting linear multivariate regression models to the data. The clinical parameters and ratios of selected parameters were first modeled as functions of nominally categorized exposure and covariates. Dependent variables that were

not normally distributed were appropriately transformed. Total serum fluorine was categorized into mutually distinct categories. Cutoff values for the categories were chosen to assure adequate numbers in each category while maintaining the fullest range of exposure values possible. Accordingly, total serum fluorine level categories were defined as the following: less than 1 ppm, greater than 1 ppm toless than 4 ppm, 4 ppm to 10 ppm, greater than 10 ppm to 15 ppm, and greater than 15 ppm. If insufficient numbers of events occurred within individual categories, the number of categories was reduced by combining adjacent categories. Additionally, models were fitted with total serum fluorine entered as a continuous variable using linear, square, square root transformations.

Age, body mass index (BMI), alcohol use and tobacco use were included in the model as potential confounders. Age was included in the models as both a categorical variable and a continuous variable. Age was grouped into four ten year age categories. Age was treated as a continuous variable using linear, square, square root, and log transformations. BMI was entered in the models as a categorical variable and as a continuous variable. BMI categories were less than 25 kg/m^2, 25-30 kg/m^2 , and greater than 30 kg/m^2. Additionally, BMI was dichotomized into obese, greater than 28 kg/m^2, and non-obese, less than or equal to 28 kg/m^2. The continuous variable was entered as linear, square, log, and square transformations. Alcohol use was categorized into 3 categories: less than 1 drink per day, greater than one to 3 drinks per day, and non response to the questionnaire item. Smoking was categorized as current nonsmokers and current smokers. A nonresponse category was not included since only two individuals were in this category. These two individuals were excluded from analyses that required smoking history. Smoking was quantified as cigarettes smoked per day. Linear, square and square root transformations of cigarettes per day were used in regression models.

The choice of the final model was somewhat subjective. For each dependent variable, other covariates were included in the final model if they were potential confounders. Other potential confounding hormones and biochemical parameters were included in the models if they produced significant changes in effect estimates.

Total serum fluorine and confounding covariates were entered into models as continuous variables. Significant nonlinear dose-response relationships were evaluated by comparing model fit and parameter estimates using categorical variables and continuous variables. Square, square root, exponential, and logarithmic transformations were used if the transformed variables produced models of superior predictive power as assessed by model fit. All two way interactions between total serum fluorine and the included covariates were evaluated. Interaction terms were included in the final model if the parameter estimate for the interaction term was significant at the alpha =.10 level.

The potential for susceptibility to confound the relationship between PFOA exposure and the assayed parameters was examined by comparing the observed prevalence of assay results outside of the reference range with the expected prevalence. The prevalence of abnormal assays was based on published reference values for the adult male US population. Reference ranges for test parameters were defined as being within 2 standard deviations above or below the mean value for the parameter. The laboratory maintains laboratory and assay specific reference range for each assay. Given that the distribution of values is approximately normal, about 2.5% of individual values are expected to fall above the upper limit and 2.5% below the lower limit. It follows that the prevalence for a high test is .025. The prevalence for a low value is .025. Using these prevalences, an expected number of tests outside of the reference range can be defined. A priori hypotheses based upon animal and in vitro studies defined the expected direction of the effect. The calculation of an observed to expected ratio allowed the estimation of the relative prevalence for a test outside of the normal range in the study subjects as compared to the general population. The 95% CI for the ratio was calculated assuming that the expected number is a constant and the observed number is a random variable with a Poisson distribution.

4. RESULTS

4.1 Cross Sectional Perfluorocarbon Physiologic Effects Study

In October 1990, at the time of the cross sectional study, the workforce at Chemolite consisted of 880 salaried and hourly employees. There were 50 men and 2 women in high exposure potential jobs. Since there were only 2 women in this group, the study was restricted to males. Forty-eight (96%) of the 50 male workers in high exposure potential jobs agreed to participate. The exact number of low exposure workers invited to participate in the study was not recorded. However, few individuals in this group refused to participate. Thus, it is estimated that over 80% of low exposure workers participated.

4.1.1 Participant Characteristics

Since frequency matching for age was used to select study participants, the overall age distribution reflected the age distribution of workers in high exposure potential jobs (Table 4.1.1). Ages ranged from 24 to 59 years, with a median age of 37 years and a mean age of 39.2 years.

Table 4.1.2 presents the alcohol and tobacco use profile of the study participants. The light drinkers category included 22 participants who reported no alcohol use. Consumption of one to three ounces of ethanol per day was reported by 20 (18.7%) participants. No participants reported drinking greater than three ounces of ethanol per day. Eight workers (7.0%) did not complete this item of the questionnaire. There were 28 (24.8%) smokers who smoked an average of 21.7 cigarettes per day. Smoking status was not available for two workers (1.8%). The association between smoking and alcohol consumption is presented in Table 4.1.3. Thirteen (15.3%) of 85 nonsmokers and seven (25.0%) of 28 smokers reported moderate drinking ($p=.24$). Table 4.1.4 displays the age distribution for alcohol and tobacco use categories. There were no significant differences in mean ages among smoking or drinking categories.

Total fluorine was not significantly correlated with age, BMI, alcohol, or tobacco use (Table 4.1.5). BMI and age were correlated (r=.26, p=.005). Alcohol use and tobacco use were not significantly correlated (r=.08; p>.7).

BMI ranged from 18.8 to 40.5 kg/m^2 with a median value of 26.3 kg/m^2 and a mean of 26.9 kg/m^2 (Table 4.1.6). Half of all workers had BMIs between 25 and 30 kg/m^2 . The mean BMI in smokers was not significantly different from that of nonsmokers (Table 4.17). The mean BMI for moderate drinkers was not significantly different from the BMI of light drinkers. Smoking status and BMI were not significantly associated (Table 4.1.8). There was a significant linear relationship between BMI and age (β=.10 SE(β)=.035). This relationship was not substantially altered after adjusting for smoking status, alcohol use, and total serum fluorine level.

4.1.2 Total Serum Fluorine

The total serum fluorine values ranged from zero to 26 with a median value of two ppm, a mean of 3.27 ppm and a standard deviation of 4.68 ppm (Table 4.1.9). The inter-assay coefficient of variation was 66% calculated from repeated assays on different days.

Twenty-three (20.0%) of 115 workers had total serum fluorine values less than one ppm. This group included eight workers values reported as zero ppm (below the limits of detection). Eighty-eight workers (76.5%) had levels less than or equal to three ppm. Six (5.2%) of 115 workers had values between 10 and 15 ppm and five (4.4%) had values greater than 15 ppm. All workers with levels greater than ten had worked in Building 15, the primary PFC production area at the Chemolite Plant.

There were no significant differences in total serum fluoride mean values among the BMI, age, alcohol use and tobacco use categories (Table 4.1.10). No statistically significant differences in mean age between total fluorine categories were observed (Table 4.1.11).

Participants with less than one ppm total fluorine smoked the least (16.3) number of cigarettes per day (Table 4.1.12). Those with one ppm to three ppm total fluorine smoked the greatest number of cigarettes per day (24.5). This difference was statistically significant ($p<.005$). As estimated in a regression model, the linear relationship between total fluorine and smoking status, adjusted for age and BMI, was small in magnitude ($\beta=0.10$, $SE(\beta)=0.062$, $p=.09$). Smokers average total serum fluorine was estimated to be 0.1 ppm higher than nonsmokers. The number of cigarettes smoked per day was weakly correlated with total serum fluorine (Table 4.1.5).

Drinking status was not associated with total fluorine (Table 4.1.13). Overall, eight (7.0%) participants did not respond to this question. Four had less than one ppm total serum fluorine.

Table 4.1.14 presents the distribution of BMI in the total fluorine categories defined previously. BMI mean values were not significant differences among the total serum fluorine categories. The linear relationship between BMI and total fluorine, adjusted for age, smoking, and alcohol use, was weak and not significant ($\beta=-.016$ $SE(\beta)=.069$, $p>.5$).

4.1.3 Hormone Assays

The intra-assay coefficient of variation (CV) for the bound and free testosterone, estradiol, TSH, LH, prolactin, and FSH assays are provided in Table 4.1.15. The estradiol assay had the highest CV, 18.3%. The prolactin assay had the lowest CV of 3.1%.

Table 4.1.16 presents the observed and expected number of hormone assays out of the assay reference range, the observed to expected (O/E) ratio , and the 95% confidence limits. The O/E ratio was significantly greater than one for estradiol, free testosterone, bound testosterone and prolactin. The O/E ratios for LH, FSH, and TSH were not significantly different from one.

The Pearson correlation coefficients among the seven hormones assayed in study participants are presented in Table 4.1.17. As expected, estradiol was

correlated with free testosterone (r=.40, p=.0001) and bound testosterone (r=.32, p=.0006). Bound testosterone was correlated with free testosterone (r=.74 p=.0001), LH (r=.28, p=.003) and FSH (r=.16, p=.04). LH and FSH were significantly correlated (r=.63, p=.0001). FSH and TSH were significantly correlated (r=.23, p=.01).

As shown in Table 4.1.18, total fluorine was significantly correlated with prolactin (r=.19, p=.045) and TSH (r=.26, p=.005). Age was negatively correlated with estradiol (r=-.25, p=.01), free testosterone (r=-.45, p=.0001), bound testosterone (r=-.24, p=.01), and prolactin (r=-.19, p=.01). Age was positively correlated with FSH (r=.33, p=.0003). As expected, BMI was negatively correlated with free and bound testosterone(r=-.26, p=.005 and r=-.36, p=.0001 respectively). BMI was correlated positively with LH (r=.20, p=.03). Alcohol consumption was significantly correlated with FSH (r=-.24 p=.01).

Bound testosterone ranged from 141 to 1192 ng/dl with a mean of 572 ng/dl and a median of 561 ng/dl (Table 4.1.19). The standard deviations were large. The mean bound testosterone values were not significantly different among the total serum fluorine groups. As expected, the mean bound testosterone decreased significantly as BMI increased. The mean bound testosterone values were significantly different among the age categories (p=.016).

There was a significant nonlinear relationship between total serum fluorine and bound testosterone (BT) in the final regression model (Table 4.1.20). Bound testosterone, which was positively associated with both LH and estradiol, decreased as both age and BMI increased. Alcohol and cigarette use were weakly associated with BT. There was a significant interaction between age and total serum fluorine. There was a negative association between bound testosterone and total serum fluorine in young workers than in older workers. In workers greater than 45 years of age, total serum fluorine was associated with a slight increase in BT. The relationship between bound testosterone and total serum fluorine is presented for four different sets of covariate value (Figure 2). Dose-response curves for bound testosterone were plotted for young, lean individuals aged 30 with BMIs of 25, young obese individuals aged 30 with BMIs of 35, middle aged lean individuals aged 50 with BMIs of 25, and middle aged

obese individuals aged 50 with BMIs of 35. Each of the relationships is for nonsmoking, light drinking men with the sample mean LH value (5.4 mU/l) and mean estradiol value (33.4 pg/ml). In 30 year old workers, bound testosterone decreased as total serum fluorine increased in both BMI groups. The dose-response relationship for 40 year old workers was approximately flat (not shown). In workers greater than 50 year of age, BT increased as total serum fluorine increased.

Total serum fluoride was not significantly associated with free testosterone (FT) (Table 4.1.21). Within BMI categories, free testosterone was highest in the less than 25 kg/m^2 group and lowest in the greater than 30 kg/m^2 category. The difference in mean FT among BMI categories was statistically significant (p=.03).

There was a significant nonlinear dose-response relationship between total serum fluorine and FT in the final regression model (Table 4.1.22). As total serum fluorine increased, free testosterone decreased. There was a significant interaction between age and total serum fluorine. Figure 4.2 illustrates the modifying effect of age on the total serum fluorine free testosterone relationship. The covariate vectors (nonsmoker, light drinker, mean LH and estradiol, age=30 and BMI=25 or 25, age=50 and BMI=25 or 35) were the same as used Figure 1. Lean or obese 50 year old men had low free testosterone (less than 9 ng/dl) for all values of total serum fluorine. In 30 year olds, free testosterone decreased asymptotically toward the 50 year old values. In this model, a 50 year old, obese, moderate drinker with any total serum fluorine level (the lower limit of assay sensitivity was approximately 1 ppm total serum fluorine) had free testosterone below nine ng/dl.

As shown in Table 4.1.23, the estradiol means in the three BMI groups were not significantly different (p=.88). As the age of participants increased, mean estradiol levels decreased. In the greater than 30 to 40 year age group, mean estradiol was 36.8 pg/ml compared to 25.9 pg/ml in the greater than 50 to 60 year age group. The age group means were significantly different (p=.018). There was a nonsignificant positive association between mean estradiol and total serum fluorine.

As shown in Table 4.1.24, estradiol and total serum fluorine were positively associated in the final regression model. Total serum fluorine followed a nonlinear relationship with estradiol. No interaction terms were statistically significant. As expected, free testosterone and estradiol were positively associated (β=.85 p=.0007). The relationship between total serum fluorine and estradiol is illustrated in Figure 3. The plotted curves depict the relationship for lean (25 kg/m^2) and obese (35 kg/m^2) male workers who were 30 years old with sample mean free testosterone and who were nonsmokers and light drinkers. As total serum fluorine increased over the observed range, estradiol increased quadratically. In obese men (BMI=35 kg/m^2) aged 30, estradiol exceeded 44 pg/ml when total serum fluorine was between 15 and 20 ppm. The highest estradiol levels were in young, obese smokers who consumed 1 to 3 ounces of ethanol per day.

LH was not significantly associated with serum fluorine, but was negatively associated with BMI (p=.003) and positively associated with smoking (p=.025), age, and BT . There was no association between total serum fluorine and FT. (Table 4.1.25, Table 4.1.26, and Figure 4).

FSH was not significantly related to total serum fluorine levels but was positively associated with age (p=.014) (Table 4.1.27, Table 4.1.28). The final regression model for FSH is illustrated in Figure 5. The relationship was essentially flat over the total fluorine range.

TSH was positively associated with total serum fluorine in both univariate and multivariate analyses (Table 4.1.29, Table 4.1.30 and Figure 7). TSH was not significantly related to age, BMI, alcohol use, smoking, and other hormones.

Prolactin was positively associated with total serum fluorine and smoking (Table 4.1.31, Table 4.1.32). Moderate drinkers had a different prolactin-total serum fluorine relationship compared to light drinkers and nonrespondents. Figure 6 illustrates the relationship of prolactin with total serum fluorine and the modifying effect of alcohol use. Total serum fluorine was weakly associated with prolactin in light and moderate drinkers. However, in moderate drinkers (1-3 oz/day), there was a positive association between prolactin and total serum fluorine.

4.1.4 Hormone Ratios

The univariate distributions of the 21 ratios are provided in Appendices 4.1 and 4.2. A table is presented for each of the 21 ratios showing the number of participants, mean ratio value with the standard deviation, median ratio value, and the range of ratio values in each of the previously defined categories of BMI, age, alcohol use, tobacco use, and total serum fluorine

Correlations between total serum fluorine (ppm), age (years), BMI (kg/m^2), alcohol use (oz/day), and cigarette consumption (cigarettes/day) and all possible ratios between E, free testosterone TF, TB, and LH are displayed in Table 4.1.33. The estradiol to bound testosterone ratio (E/TB) and estradiol to free testosterone ratio (E/TF) were significantly correlated with BMI (r=.32, p=.001 and r=.27, p=.004 respectively). The estradiol to luteinizing hormone ratio (E/LH) was negatively correlated with age (r=-.26, p=.005), and positively correlated with BMI (r=.18, p=.06). The bound testosterone to luteinizing hormone ratio (TB/LH) followed a different pattern as compared to E/LH. The correlation coefficient between TB/LH and age was -.32 (p=.001) while the coefficient between TB/LH and BMI was -.14, (p=.13). The free testosterone to luteinizing hormone ratio (TF/LH) had the strongest correlation with age (r=-.40, p=.0001) but was not significantly correlated with BMI. The bound testosterone to free testosterone ratio (TB/TF) followed a unique pattern. TB/TF was positively correlated with age (r=.24, p=.01), and negatively correlated with BMI (r=-.16,p=.08).

Prolactin ratios with bound testosterone (TB/P), free testosterone (TF/P), estradiol (E/P), follicle stimulating hormone (FSH/P), luteinizing hormone (P/LH), and thyroid stimulating hormone (P/TSH) are presented in Table 4.1.34. None of the prolactin-hormone ratios were significantly correlated with total serum fluorine or BMI. All except P/TSH were significantly correlated with cigarette consumption.

Table 4.1.35 presents the Pearson correlation coefficients for the bound testosterone to thyroid stimulating hormone (TB/TSH) ratio, the free testosterone to thyroid stimulating hormone (TF/TSH), and the estradiol to thyroid stimulating

hormone (E/TSH). Total serum fluorine and TF/TSH were negatively correlated ($r=-.18, p=.05$). All three ratios were significantly and negatively correlated with age. TB/TSH and TF/TSH were negatively correlated with BMI, ($r=-.24$ $p=.01$ and $r=-.23$, $p=01$ respectively).

The Pearson correlation coefficients for the bound testosterone to follicle stimulating hormone (TB/FSH) ratio, the free testosterone to follicle stimulating hormone (TF/FSH), and the estradiol to follicle stimulating hormone (E/FSH) are provided in Table 4.1.36. Age was the only covariate that was significantly correlated with the three ratios.

The correlation coefficients for selected ratios between pituitary glycoprotein hormones, TSH, LH, and LH, are presented in Table 4.1.37. The thyroid stimulating hormone to follicle stimulating hormone (TSH/FSH), the thyroid stimulating hormone to luteinizing hormone (TSH/LH), and the follicle stimulating hormone to luteinizing hormone (FSH/LH) are provided. Age was significantly correlated with the FSH/LH ratio and the TSH/FSH ratio. Alcohol consumption was correlated with both TSH/FSH and TSH/LH.

As shown in the final regression models, the TB/TF ratio increased as total serum fluorine increased (Tables 4.38 and 4.39). Alcohol consumption, cigarette consumption, estradiol, prolactin, and TSH were not significantly related to the TB/TF ratio in either model. These covariates do not substantially alter the estimated relationship between total serum fluorine and TB/TF ratio when included in the regression model. The quadratic increase of the TB/TF ratio over the observed range of total serum fluorine is illustrated in Figure 4.8. The covariates used were: nonsmoker, less than one ounce of alcohol consumed per day, 30 years of age, and a BMI of 30 kg/m^2.

Table 4.1.40 presents the full regression model for the estradiol to bound testosterone ratio (E/TB). Total serum fluorine was not significantly associated with the E/TB ratio. BMI was a determinant of the E/TB ratio. Free testosterone was negatively related to the E/TB ratio.

The full regression model for estradiol to free testosterone ratio (E/TF) is displayed in Table 4.1.41. There was a significant positive dose-response relationship between the E/TF ratio and total serum fluorine. Although the dose-response relationship for free testosterone was modified by age, the dose-response relationship for the ratio was not modified by age.

As shown in Tables 4.1.42, 4.1.43 and 4.1.44, total serum fluorine was not significantly associated with E/LH and TB/LH, but was positively association with the TF/LH ratio (ß=-.05, p=.09). Bound testosterone and FSH were associated with the TF/LH ratio (ß=.003, p=.0001) and (ß=-.33, p=.0001).

Cigarette consumption and free testosterone were strongly and significantly related to the TB/P ratio (ß=1.49, p=.02 and ß=3.93, p=.008 respectively) (Table 4.1.45). Cigarette consumption and bound testosterone were significantly related to the TF/P ratio (ß=.04, p=.03 and ß=.002, p=.03 respectively) (Table 4.1.46). Only cigarette consumption was significantly related to the E/P ratio (ß=.10, p=.005) (Table 4.1.47).

Tables 4.48 through 4.50 present full regression models for the ratios of prolactin to FSH (P/FSH), prolactin to LH (P/LH), and prolactin to TSH (P/TSH). In each of the three regression models total serum fluorine was positively and significantly associated with the prolactin-hormone ratio. Moderate drinkers had a significantly different ratio total serum fluorine dose-response relationship compared to the relationships in light drinker and nonrespondents.

The full regression models for the glycoprotein hormone ratios are presented in Table 4.1.51 to 4.1.59. As shown in table 4.1.52, total serum fluorine was significantly related to TF/TSH (ß=-.28, p=.03) and bound testosterone and FSH were significantly related to the TF/TSH ratio (ß=.01, p=.006 and ß=.68, p=.04 respectively). Total serum fluorine was not significantly associated with the other glycoprotein hormone ratios.

4.1.5 Cholesterol, Low Density Lipoprotein, High Density Lipoprotein, And Triglycerides

Table 4.1.60 provides the correlation coefficients for serum lipids, specifically cholesterol, low density lipoprotein (LDL), and high density lipoprotein (HDL), with total serum fluorine, age, BMI, alcohol consumption, and cigarette consumption. Total serum fluorine was not significantly correlated with cholesterol, LDL, HDL, or triglycerides. Cholesterol and triglycerides were correlated with age (r=.25, p=.008 and r=.19, p=.04, respectively), and BMI (r=.19, p=.04 and r=.27, p=.004, respectively). Cigarette smoking was positively and significantly correlated with cholesterol (r=.35, p=.0001), LDL (r=.28, p=.002), and triglycerides (r=.19, p=.04). HDL was not significantly correlated with any variable, although the correlation with alcohol consumption was suggestive (r=.18, p=.06).

Total fluorine was not significantly associated with cholesterol, LDL or triglycerides (Tables 4.1.61, Table 4.1.62, Table 4.1.64). Smoking, age, and GGT were positively and significantly associated with cholesterol. Smoking and prolactin were positively and significantly associated with LDL. Smoking and free testosterone were positively associated and bound testosterone was negatively associated with triglycerides.

The final regression model for HDL, displayed in Table 4.1.63, presents a different picture. HDL decreased as total fluorine increased in moderate drinkers. In light drinkers, there was a negligible change in HDL as total fluorine increased. Self-reported moderate alcohol consumption was positively associated with HDL. Additionally, bound testosterone was positively associated with HDL, while free testosterone was negatively associated.

4.1.6 Hepatic Parameters: Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), Alkaline Phosphatase (AKPH), Gamma Glutamyl Transferase (GGT).

Table 4.1.65 presents the correlation coefficients between the hepatic parameters, SGOT, SGPT, GGT, AKPH, and total serum fluorine, age, BMI, alcohol consumption, and cigarette consumption. The hepatic parameters were not significantly correlated with total serum fluorine. SGOT was not significantly correlated with any of the participant characteristics. SGPT and GGT were correlated significantly only with BMI (r=.20, p=.02 and r=.27, p=.004

respectively). AKPH was significantly correlated with age, BMI, alcohol consumption, and cigarette consumption.

The correlation coefficients between the hepatic parameters and cholesterol, LDL, HDL, triglycerides, estradiol, TF, TB, and prolactin are displayed in Table 4.1.66. SGOT and AKPH were significantly correlated with prolactin. SGPT was correlated with cholesterol and triglycerides. GGT was correlated with cholesterol, triglycerides, and free testosterone. As expected, SGOT, SGPT, and GGT were highly correlated (Table 4.1.67). AKPH was only correlated with GGT.

The SGOT, SGPT, GGT, and AKPH mean values were not significantly different among the five total serum fluorine categories (Table 4.1.68). SGOT and SGPT mean values were not significantly different for BMI, age, alcohol use, and smoking (Tables 4.1.69 to 4.1.72) . Mean GGT was significantly higher in the greater than thirty BMI group (p=.03). As shown in Table 4.1.72, mean and median AKPH values were significantly higher in smokers compared to nonsmokers (p=.012).

Tables 4.1.73 A, B, and C present three linear multiple regression models for SGOT. In non-obese workers (BMI=25), SGOT decreased as total fluorine increased. In obese workers (BMI= 35), the association between total serum fluorine and SGOT was in the opposite direction. Model 2 included GGT as a covariate (Table 4.1.73 B). The association between total fluorine and SGOT, as well as the effect modification by BMI, were present after adjusting for GGT. When SGPT was included in the regression model (Table 4.1.73 C), the association between total fluorine and SGOT was weak and nonsignificant . The effect modification by BMI was no longer present. AKPH had little effect on the regression estimates when included in the model.

Three linear multiple regression models for SGPT are provided in Tables 4.1.74 A, B, and C. In non-obese workers (BMI=25), SGPT decreased as total fluorine increased. However, in obese workers (BMI= 35), the association between total serum fluorine and SGPT was in the opposite direction. Little change occurred in the estimates after adjusting for GGT. As seen in Table 4.1.74 C, the association was significant, although weaker in strength, after adjusting for SGOT. The effect

modification by BMI was present. When AKPH was included in the model, effect estimates did not change significantly.

The final regression models for GGT, provided in Tables 4.75 A, B, and C, present a different picture. GGT decreased as total fluorine increased in moderate drinkers. In light drinkers, GGT decreased less steeply as total fluorine increased. Controlling for SGOT and SGPT (model 2 and 3) did not significantly alter the relationship between total fluorine and GGT. Moderate alcohol consumption was positively associated with GGT.

Table 4.1.76 presents the final regression model for AKPH. In nonsmokers, total serum fluorine was negatively associated with AKPH. As the number of cigarettes smoked per day increased to more than five per day, AKPH increased as total serum fluorine increased.

4.1.7 Hematology Parameters: Hemoglobin, White Blood Count, Polymorphonuclear Leukocyte Count, Band Count, Eosinophil Count, Lymphocyte Count, Monocyte Count, Platelet Count, And Basophil Count.

Table 4.1.77 presents the correlation coefficients between the nine hematology parameters and total serum fluorine, age, BMI, alcohol use, and cigarette consumption. The only parameter that was significantly correlated with total serum fluorine was lymphocyte count (r=.19, p=.04). Monocyte count was correlated with BMI (r=-.22, p=.04) and alcohol consumption, (r=-.21, p=.03). All the parameters, except the basophil and band counts, were strongly associated with cigarette consumption. Alcohol consumption was correlated with hemoglobin, (r=-.20, p=.04), and band count (r=.26, p=.005).

The final regression models for hemoglobin and the erythrocyte indices, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), are presented in Tables 4.1.78, 4.1.79, and 4.1.80 respectively. Total serum fluorine was significantly associated with hemoglobin.. The association hemoglobin and MCV were modified by smoking. In smokers who smoked seven or more cigarettes per day, hemaglobin and MCV increased significantly as total fluorine increased. In nonsmokers, hemaglobin and MCVdecreased as total fluorine

increased . The association of total fluorine with MCH was modified by smoking and by alcohol use. The increase in MCH as total fluorine increased was enhanced with increased smoking. In light drinkers, total serum fluorine had a weak association with MCH. In moderate drinkers, MCH increased as total fluorine increased. There was a positive association of both MCH and MCV with alcohol consumption. None of the estimated associations are of clinically significant magnitude over the range of total fluorine values.

The white blood cell count (WBC) increased significantly in nonrespondents as total fluorine increased above 2ppm, increased less in moderate drinkers, and increased the least in light drinkers (Table 4.1.81). As expected, cigarette smoking intensity was positively associated with WBC. PMN increased significantly in alcohol use nonrespondents as total fluorine increased and increased less steeply in moderate drinkers (Table 4.1.82). In light drinkers, total serum fluoride above 10 ppm was associated with a decreased in PMN. Cigarette smoking was positively associated with PMN. As shown in Table 4.1.83, the final regression models for band count provides little evidence that total fluorine was associated with band count. Moderate alcohol use was estimated to reduce the band count. Smoking was positively associated with band count.

The negative association between total fluorine and lymphocyte count was modified by adiposity, alcohol consumption, and cigarette smoking (Table 4.1.84). The decrease in lymphocyte count was smaller as BMI increased. The decrease in lymphocyte count associated with total fluorine above 3 ppm was greater in moderate drinkers compared to nonrespondents. As cigarette consumption increased, the decrease in lymphocyte count increased.

The positive association between total fluorine and monocyte count (MONO) was modified by adiposity (Table 4.1.85). As BMI increased, the association with MONO was weaker. Cigarette smoking and LH were positively associated with MONO. Alcohol consumption was negatively associated with MONO. The association between total fluorine and eosinophil count (EOS) was negative for nonsmokers, but was positive as more than ten cigarettes per day were smoked

(Table 4.1.86). As smoking increased, the PFOA associated decrease in BASO was smaller (Table 4.1.88).

The association between total fluorine and platelet count (PLAT) was modified by adiposity and cigarette smoking intensity (Table 4.1.87.). In lean participants (BMI=25), PLAT increased as total fluorine increased. In obese participants (BMI=40), the PLAT decreased as total fluorine increased. As smoking increased, the rate of increase in PLAT associated with total fluorine above 10 ppm decreased.

4.1.8 Summary Of Results

The serum fluorine levels in Chemolite workers were 20-100 times higher than expected in workers not directly involved in PFOA production. All workers with levels above 10 ppm fluorine work in PFOA production areas. Smoking was associated with a small increase in serum fluorine. Age was not associated with serum fluorine levels. The two women employed in the PFOA production areas had total serum fluorine levels similar to men.

Alcohol use, smoking, age, BMI, and hormones had the expected associations with peripheral leukocyte counts, hematology parameters, cholesterol, HDL, LDL, and hepatic enzymes.

The main hormone results are:

1. The number of male workers with hormone values outside of the laboratory reference range was greater than expected for estradiol, free testosterone, bound testosterone, and prolactin.
2. Total serum fluorine was negatively associated with free testosterone and positively associated with estradiol. No association was noted between total serum fluorine and LH.
3. E/TF and TB/TF, but not E/TB, were positively associated with total serum fluorine.
4. E/LH and TB/LH were not associated with total serum fluorine. However, the relationship between total serum fluorine and TF/LH was suggestive.

5. TSH was positively associated with total serum fluorine. TF/TSH was negatively associated with total serum fluorine; TB/TSH and E/TSH were not.
6. Prolactin and total serum fluorine were positively associated in moderate drinkers, but not in light drinkers.
7. P/FSH, P/LH, P/TSH were positively associated with total serum fluorine. TB/P, TF/P, and E/P were not associated with total serum fluorine

The main hepatic parameter results are:

1. The increase in SGOT and SGPT levels associated with adiposity was enhanced by total serum fluorine.
2. The induction of GGT by alcohol was decreased as total serum fluorine increased.
3. The induction of AKPH by smoking was increased by increasing levels of total serum fluorine.

The main cholesterol and lipoprotein results are:

1. Cholesterol and triglyceride levels were not associated with total serum fluorine.
2. LDL was not associated with total serum fluorine.
3. The positive association between moderate alcohol use and HDL levels was reduced as total serum fluorine increased.

The main hematology parameter and peripheral leukocyte count results are:

1. The effect of smoking on hemoglobin and MCV was enhanced by total serum fluorine.
2. Total serum fluorine was negatively associated with all peripheral leukocyte counts except PMNs and MONOs, which were positively associated.
3. The associations between cell counts and total serum fluorine were modified by smoking, drinking, and adiposity.

4.2 The 1990 Chemolite Retrospective Cohort Mortality Study

A total of 3,537 individuals who were employed at the Chemolite plant between January 1, 1947 and December 31, 1983 were identified from company records. The cohort consisted of 2,788 (79%) male and 749 (21%) females employees (Tables 4.2.1 and 4.2.2). The majority of women (67.3%) never worked in the Chemical Division. Of the 19,309 person years (PY) observed for women, 68.8% occurred in those who were never employed in the Chemical Division. The mean follow-up for women was 25.8 years in the overall cohort, 24.6 years in the Chemical Division (CD) cohort, and 26.4 years in the non-CD cohort. The distribution of follow-up periods was similar in the women's CD and non-CD cohorts.

The women's mean age at first employment was 27.6 years. Sixty-eight percent were less than 30 years old at employment; 9.7% were older than 40 at first employment at Chemolite. The CD cohort was slightly older than the non-CD cohort. The CD and non-CD distributions of latency times were not statistically different ($p=.66$). The mean duration of employment for women was 8.7 years and ranged from six months to 41.4 years. The distribution of years of employment was significantly different for CD and non-CD women ($p<.0001$). Of non-CD women, 11.9% were employed for more than twenty years. Of 245 women in the CD cohort, 51(21.1%) were employed for more than twenty years.

As shown in Table 4.2.2, the 2,788 men who were ever employed for more than six months at Chemolite contributed a total of 71,117.7 PY which was about equally divided between the CD and non-CD cohorts. The mean follow-up for the overall male cohort was 25.5 years. The distribution of follow-up periods and distribution of year of first employment was similar in the male CD and non-CD cohorts. The average age at death was higher in the male non-CD group, 58.1 years, compared to the CD group, 54.2 years. The duration of employment for men (mean 13.6 years, median 9.8 years) was longer than for women. The distribution of years of employment was significantly different for CD and non-CD men ($p<.0001$). Of non-CD men, 25.5% were employed for longer than twenty

years. Of men in the CD cohort, 38.0% were employed for longer than twenty years.

Vital status was obtained for 100% of the women's cohort (Table 4.2.3). Among the 749 women there were 50 deaths; 11 in the CD cohort and 39 in the non-CD cohort. Vital status was obtained for 100% of the men's cohort. Among the 2788 men there were 348 deaths; 148 deaths in the CD group and 200 in the non-CD group. Six individuals who had employment records that were missing information were excluded from the cohort and their vital status was not ascertained. Death certificates were obtained for 99.5% of deaths. Two deaths occurred outside the U.S. and causes of death were ascertained by other means.

4.2.1 Standardized Mortality Ratios (SMRs)

4.2.1.1 SMRs For Women

The numbers of deaths, the SMRs and 95% confidence intervals (CI) among women in the 1947-1989 follow-up period are shown in Table 4.2.5. The SMRs for all causes of death (SMR=.75, 95% CI .56-.99), and cancer (SMR=.71, 95% CI .42-1.14) were significantly lower than expected in comparison to national rates. No association was found with duration of employment or latency for deaths from all causes, cancer, and cardiovascular diseases (Tables 4.2.6 and 4.2.7). SMRs for CD women and non-CD women are displayed in Table 4.2.8. The estimated SMR for the CD cohort of women were less than expected. In CD women, the all causes SMR was .46 (95% CI .23,.86) and the cancer SMR was .31 (95% CI .07,1.05). The SMRs for the non-CD women were closer to unity.

4.2.1.2 SMRs For Men

The number of male deaths, the expected number of male deaths based on U.S. national white male rates, and age and calendar period adjusted SMRs with associated 95% CIs are presented in Table 4.2.9. The SMR for all causes (.73, 95% CI .66,.81), for cardiovascular diseases (SMR=.71, 95% CI 60,.48), for all gastrointestinal (GI) diseases (.50,95% CI .26,.87) and for all respiratory diseases (.50,95% CI .27,.86) were significantly less than one. None of the

cause-specific SMRs were large nor were the estimates significantly different from one. As shown in Table 4.2.10, the results were similar when the expected numbers of male deaths was based on Minnesota white male rates.

Table 4.2.11, Table 4.2.12, and Table 4.2.13 present adjusted SMRs and 95% CI for males based on Minnesota mortality rates for three latency intervals 10, 15, and 20 years respectively. The three latency intervals the all causes SMR ranged from .75 to .77. For all cancers, SMRs ranged from 1.06 to 1.12 and were nonsignificant. Among men there was no association between any cause of death and duration of employment (Table 4.2.14, Table 4.2.15, and Table 4.2.16).

Table 4.2.17 and 4.2.18 display the SMRs and 95% CI for CD and non-CD male workers. The all causes SMRs were .69 (.59,.79) for the non CD group and .86 (.72,1.01) for the CD group. The SMRs for prostate cancer, based on a comparison with Minnesota population rates, were 2.03 (95% CI .55,4.59) in the CD group and .58 (95% CI .07,2.09) in the non-CD cohort. There were 4 observed deaths from prostate cancer compared to 2 expected in the CD group. The latency analysis for non-CD and CD men are presented in Tables 4.2.19 and 4.2.20. There was no associations between any cause of death and latency in either group.

As shown in Table 4.2.21 and 4.2.22, male CD cohort members with more than 10 or more than 20 years of employment had SMRs that were less than one for all causes of death, all malignancy, cardiovascular diseases and all respiratory diseases. Among male non-CD cohort members with more than ten years of employment or more 20 years of employment, the SMRs for all causes, cardiovascular disease and all respiratory diseases were significantly less than expected (Table 4.2.23 and 4.2.24). There was no association of any cause of death with duration of employment at Chemolite in either CD or non-CD groups.

4.2.2 Standardized Rate Ratios (SRRs)

Age adjusted standardized rate ratios (SRRs) were calculated for all causes, all cancer, and cardiovascular diseases mortality comparing men employed at the

plant for ten years or more to men employed for less than ten years. The SRRs are presented in Table 4.2.25. The 95% CIs for all causes, all cancer, and all cardiovascular diseases were wide and include one. Confounding variables such as year of first employment and length of follow-up were not controlled in this analysis due to small numbers and unstable rates within the large number of strata.

Table 4.2.26 presents the age adjusted SRRs for all causes, all cancers, lung cancer, GI cancer, and all cardiovascular diseases mortality comparing men ever employed in the CD with men never employed in the CD. All SRRs were slightly greater than one, however, none was statistically significant.

4.2.3 Mantel- Relative Risks (RR_{MH})

Age stratified RR_{MH}, contrasting the rates in men ever employed in the CD compared to the rates in men never employed in the CD, were calculated for all causes, all cancer, and all cardiovascular diseases mortality and are displayed in Table 4.2.27. The estimated RR for CD employment versus non-CD employment did not follow a monotonic pattern and the 95% CIs include one for each of the three endpoints.

Table 4.2.28 presents the RR_{MH} for men employed for less than ten years to those employed for more than ten years. The all causes RR_{MH} (2.16, 95% CI 1.52, 2.70) in the 30 to 39 year age at first employment strata was reflected in both the RR_{MH} for all cancers (1.75, 95% CI.95,3.21) and cardiovascular diseases (3.53 , 95% CI 1.68,6.21). The RR_{MH} were not adjusted for important time covariates such as the year of first employment.

4.2.4 Proportional Hazard Regression Model Relative Risk Estimates

4.2.4.1 Proportional Hazard Models For Male Workers

Table 4.2.29 to 4.2.36 show the final proportional hazard (PH) model for death from all causes, cardiovascular diseases, all cancers, lung cancer, GI cancer, prostate cancer, pancreatic cancer, and diabetes among the 2788 male workers

ever employed at Chemolite for greater than six months. There was no evidence for violation of the PH assumptions or for significant nonlinear associations between the independent variables and mortality. As expected, age at first employment was positively associated with all causes of death. The RR for a one year increase in age at first employment was 1.082 (95% CI 1.069,1.094). Year of first employment and duration of employment were negatively associated with all causes mortality. The risk of death associated with months in the Chemical Division was small and nonsignificant.

For cardiovascular diseases mortality, the RR for a one year increase in age at first employment was 1.126 (95% CI 1.069,1.094). Year of first employment was negatively associated with cardiovascular diseases mortality. Time in the CD was not associated with death from cardiovascular diseases.

Age at first employment was positively associated with cancer mortality. The RR for a one year increase in age of employment was 1.08 (95% CI 1.06,1.10). Duration of employment was negatively associated with cancer. The RR was .972 (95% CI.96,.99) for a one year increase in employment. There was no association of cancer mortality with employment time in the CD.

The final prostate cancer mortality proportional hazard model for male cohort members is shown in Table 4.2.34. Time in the Chemical Division was positively and significantly associated with prostate cancer mortality. The relative risk for a one year increase in CD employment time was 1.13 (95% CI 1.01,1.43). Age at first employment was positively associated with prostate cancer mortality risk. A one year increase in age at first employment was associated with a RR of 1.09 (95% CI .99,1.19). The RR for lung cancer mortality was 1.07 (95% CI .03,1.12) for a one year increase in age of employment. Months in the chemical division was not significantly associated with lung cancer mortality. Table 4.2.33 shows the final proportional hazard (PH) model for all GI cancer mortality. The estimated RR for a one year increase in age at first employment was 1.14 (95% CI 1.09,1.19). Year of first employment, duration of employment and time employed in the CD were not associated with GI cancer risk. Age at first employment was positively associated with pancreatic cancer mortality. The other covariates were weakly associated with pancreatic cancer risk and were

not significantly different from one. A one year increase in age at first employment was positively associated with diabetes mortality (RR = 1.10, 95% CI 1.01,1.19).

4.2.4.2 Proportional Hazard Models For Female Workers

Table 4.2.37, 4.2.38 and 4.2.39 show the final PH model for death from all causes, cardiovascular diseases and all cancers among the 749 female cohort members. Age at first employment was positively associated with all causes mortality. The RR for all causes of death among women employed for two to ten years (3.72) and among women employed for greater than ten years (2.33) were significantly greater than the all causes mortality in women employed for less than two years. Time in the CD was not related to mortality. The RR for death from cardiovascular diseases associated with a one year increase in age at first employment was 1.13 (1.07,1.18). The year at first employment, duration of employment, and time in the CD were not significantly associated with female cardiovascular diseases mortality. The RR for death from cancer was associated with age at first employment. A one year increase in age at first employment increase the RR for death from cancer (1.09 (1.04,1.14). The year at first employment, duration of employment, and time in the chemical division were weakly and non-significantly associated with female cancer mortality.