OXIDATIVE STRESS AND NUTRITION IN LUNG AND LIVER TRANSPLANT RECIPIENTS

by

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Institute of Medical Science

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ABSTRACT OF THESIS

Oxidative Stress and Nutrition in Lung and Liver Transplant Recipients
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Transplantation is an acceptable treatment for end-stage lung and liver disease patients. In lung transplantation, long-term survival is limited due to Bronchiolitis Obliterans Syndrome (BOS) and in liver transplantation, Hepatitis C Virus (HCV) disease recurrence significantly impacts long-term survival. Treatment options are limited and often not successful. It is therefore important to conduct research on the factors contributing to the pathogenesis and disease severity of BOS and HCV to improve our understanding of the mechanisms and potentially reduce morbidity and mortality. Several factors may play a role. The focus of this thesis is to assess the role of Oxidative Stress (OxS) and nutrition on these patient populations. BOS is a frequent complication of lung transplantation. OxS may contribute to its pathogenesis and induce further tissue injury and inflammation. OxS can be influenced by several factors including nutrition. The cross-sectional study showed that BOS lung recipients have elevated markers of OxS in their Bronchoalveolar Lavage Fluid (BALF) compared to those without BOS. However, there was no difference in nutritional factors potentially affecting OxS.
HCV reinfection post transplant is universal, significantly increasing morbidity and mortality. OxS is involved in the pathogenesis of chronic HCV but its role in HCV disease recurrence is unknown. A first study determined whether HCV liver recipients (HCV-LT) were more oxidatively stressed when compared to controls or HCV non-transplant patients. A second study assessed OxS at six-and 12 months post transplant and compared results between those with and without recurrence.

The results showed that HCV-LT were more oxidatively stressed, vitamin A intakes were significantly lower and plasma gamma-tocopherol was significantly higher in HCV-LT. Additionally, those with recurrence were more oxidatively stressed at six-months (before recurrence) and 12 months compared to those without recurrence. No differences were seen regarding nutrition parameters. These results suggest that OxS is present in transplant recipients but that nutritional factors do not play a significant role. Other causes of OxS likely play a more significant role such as the presence of inflammation due to immunological reactions associated with BOS and the generation of reactive oxygen species (ROS/RNS) seen in patients with HCV disease.
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This research project would of course, not have been possible without the enthusiasm and commitment of the lung and liver transplant participants, a special thanks is extended to all of them.

A special thank you to Emma Tucker for her statistical support.
STATEMENT OF CO-AUTHORSHIP

I participated in all of the research projects described, from conception to completion. This involved designing and conducting the study, as well as collecting, analyzing and interpreting the data. From these studies, I wrote one review of the literature and 3 manuscripts, which are either published, in press or under review:

1. **Review article: Lung transplantation: does OxS contribute to the development of BOS?** Transplantation Reviews 2009; April 23(2): 103-110.

2. **OxS and Nutritional Intakes in Lung Patients with Bronchiolitis Obliterans Syndrome (BOS).** Transplantation Proceedings 2009; 41 (9):3833.

3. **OxS and Nutrition in Hepatitis C liver recipients, Controls and HCV non-transplant patients.** Accepted, Transplantation Proceedings, June 2010.

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LIST OF ABBREVIATIONS

\(^1\text{O}_2\) Singlet Oxygen
ACR Acute cellular rejection
BAL Bronchoalveolar lavage
BALF Bronchoalveolar lavage fluid
BOS Bronchiolitis Obliterans Syndrome
CAT Catalase
CRP C-reactive protein
DRI Dietary Reference Intakes
EAR Estimated Adequate Requirements
ELF Epithelial lining fluid
FEV\(_1\) Forced Expired Ventilation in one second
FR Free Radical
FVC  Forced Vital Capacity
GSH  Glutathione
GSH-Px  Glutathione peroxidase
GSSG  Oxidized glutathione
HAI  Histological activity index
HC  Healthy Controls
HCV  Hepatitis C virus
HCV-LT  Hepatitis C liver recipients
H₂O₂  Hydrogen Peroxide
HOCl  Hypochlorous Acid
IRI  Ischemia-reperfusion injury
ISHLT  International Society of Heart and Lung Transplantation
MDA  Malondialdehyde
Met O  Oxidized methione
MPO  Myeloperoxidase
NFκβ  Nuclear Factor kappa beta
NO•  Nitric Oxide
O₂•−  Superoxide radical
OH•  Hydroxyl radical
ONOO•  Peroxynitrite
OxS  Oxidative Stress
PMN  Polymorphonuclear leukocytes
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>UNOS</td>
<td>United Network for Organ Sharing</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
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IF WE KNEW WHAT WE WERE DOING IT WOULDN’T BE RESEARCH.

ALBERT EINSTEIN
PERSONAL INTRODUCTION

My initial interest as a dietitian in the transplant unit was to provide adequate nutrition to patients pre and post transplantation. I then developed an interest in research and as part of my Masters Degree, I demonstrated that low Body Mass Index (BMI) was an independent risk factor for morbidity and mortality following lung transplantation (1).

For my doctorate degree, I have continued to develop as a Clinician-Scientist with an interest in assessing the role of OxS and nutrition in lung transplantation in relation to BOS, and in liver transplantation in relation to HCV disease recurrence. Therefore, my thesis will be presented in two parts. The first (subsequently referred to as Part A) refers to lung transplantation and the second (referred to as Part B) refers to liver transplantation.
Overview of Transplantation and OxS

Transplantation is the only treatment of choice for End-stage lung and liver disease. This procedure is labor-intensive, expensive and is driven by the availability of organ donation. Considering the ongoing significant organ donor shortage, it is important to ensure that each transplant candidate will survive and achieve the maximum long-term outcome. Unfortunately, there are still challenging long-term complications associated with certain groups of transplant recipients and research is ongoing to maximize medical treatment.

In lung transplantation, long-term outcome is hampered by the development of Bronchiolitis Obliterans Syndrome (BOS). Although the survival rate at one year is 80%, this rapidly decreases to 50% at five-year post transplant. Similarly, for liver transplant recipients there is increased morbidity and mortality associated with HCV disease recurrence in those transplanted for HCV disease.

Medical and pharmacological approaches to prevent these long-term complications are improving but the success is limited. The main reason for this limited success is the complex pathogenesis of BOS and HCV disease recurrence, which can be associated with Ischemia-reperfusion injury (IRI), immunological reactions, rejection, inflammation and concomitant infection. One other potential contributor is OxS.
Oxidative Stress (OxS)

The role of Oxygen ($O_2$) is double-edged. It is utilized in the metabolic processes which provide energy for cell functions, and in this process, generates toxic free radicals (2). Under normal conditions these free radicals, which include reactive oxygen species (ROS) and reactive nitrogen species (RNS), are efficiently scavenged by the antioxidant defense system (3). However, there are a number of chronic inflammatory conditions such as aging (4) atherosclerotic heart disease (5), Alzheimer’s disease (6) as well as a variety of lung (7) and liver diseases (8, 9) whereby the production of free radicals overwhelms the antioxidant defense system leading to a condition known as OxS (Figure 1).

Physiological levels of ROS/RNS are essential for cell differentiation; cell growth; cell apoptosis and immunity against invading microorganisms (10-12). However, when these free radicals overwhelm the antioxidant defense system, OxS occurs. ROS/RNS are increased during processes such as immunological reactions, inflammation, infection and IRI (Figure 2). On the other hand, antioxidant levels can be reduced by the chronicity and magnitude of these processes and can be influenced by dietary intake of certain types of fat and antioxidant micronutrients such as vitamin E, ascorbic acid carotenoids and selenium.
**Measuring OxS**

OxS can be assessed by various laboratory analyses, for example by measuring by-products of lipid, protein and DNA oxidation. These include, lipid peroxidation metabolites such as plasma/tissue malondialdehyde (MDA) and 8-isoprostanes (13); protein oxidation parameters such as protein carbonyls, total thiols, advanced oxidation protein products and nitrotyrosine (14,15) and measures of DNA damage, such as DNA strand breaks and guanine oxidation products (8-OHdG) (16,17).

The antioxidant system can also be assessed by measuring antioxidant enzymes like glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) or by analysis of micronutrients such as antioxidant Vitamin E, C or carotenoids. Several of these tests will be discussed in a later section.

Considering that OxS may play a role in the pathogenesis of BOS in lung recipients and HCV disease recurrence in liver recipients and considering that OxS can be associated with nutritional factors, the aim of this research project was to document the presence of OxS and assess the antioxidant status and dietary intake of these transplant patients.
Figure 1. Oxidative Stress

- PUFA*
- Superoxide Radicals
- Hydroxyl Radicals
- Peroxyl Radicals
- Hydrogen Peroxide
- Singlet Oxygen

Antioxidant Defense

Vitamin E
Vitamin C
Carotenoids
Glutathione
Selenium

*PUFA, Polyunsaturated fatty acids
**Figure 2.0 Effects of Oxidative Stress**

(IRI injury/infection/rejection)
CHAPTER I: INTRODUCTION

I. A. REVIEW ARTICLE: LUNG TRANSPLANTATION: DOES OXS CONTRIBUTE TO THE DEVELOPMENT OF BOS?

by

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Madill et al. Transplantation Reviews 2009: 23 (2):103-110
Lung transplantation is an acceptable treatment for patients with end-stage lung disease. The most common indications for transplantation are COPD/Emphysema (EMP) (37%), Idiopathic Pulmonary Fibrosis (17%) (IPF) and Cystic Fibrosis (CF) (16%) (18). As of June 2006, 17,616 lung transplants (8,316 single, and 9,300 bilateral) had been performed internationally (International Society of Heart and Lung Transplantation (ISHLT) Registry) (18). The excellent one-year survival rate of 80% is due to advanced medical and surgical care, as well as improvements in immunosuppressive and anti-infectious medications. However, long-term survival is significantly limited to 50% primarily due to Bronchiolitis Obliterans Syndrome (BOS) (19).

I.A.2 BOS definition

BOS is described as an irreversible, progressive airflow obstruction and a sustained drop in forced expired ventilation in one second (FEV$_1$). Bronchiolitis obliterans is often called the ‘vanishing airway disease’, and is described as a fibrotic process where scarring and the obliteration of the terminal bronchiole lumen result in progressive narrowing of the lumen constricting airflow (20, 21). The specific marker to identify BOS is decreased FEV$_1$, based on the ISHLT BOS grading system and outlined in I.A.2. Table 1

I.A.2.1 Table 1   BOS Staging System
BOS is classified according to the current International Society of Heart and Lung Transplant (ISHLT) staging system (22).

- **BOS 0** defined as a FEV\(_1\) > 90% of baseline and \(\text{FEF}_{25-75}\) > 75% of baseline
- **BOS 0p** defined as a FEV\(_1\) 81% to 90% of baseline and/or \(\text{FEF}_{25-75}\) ≤ 75% of baseline
- **BOS 1** defined as FEV\(_1\) 66% to 80% of baseline
- **BOS 2** defined as FEV\(_1\) 51% to 65% of baseline
- **BOS 3** defined as FEV\(_1\) 50% or less of baseline

I.A.3. **BOS Pathogenesis**

I.A.3.1 Role of Immunity in BOS pathogenesis

BOS is the pathophysiological manifestation of chronic rejection involving both alloimmunological and non-alloimmunological processes (23) I.A.3.1 Figure 1.0. The bulk of scientific evidence indicates that the alloimmunological injury, including rejection and HLA mismatching, directed towards epithelial and endothelial cells, plays a key role (24). However, the non-alloimmunological inflammatory response resulting from inhaled agents, infections, ischemia and gastro-esophageal reflux disease (GERD), also contribute to the injury. These conditions are associated with the release of inflammatory mediators from a variety of cells such as epithelial cells, monocytes/macrophages, neutrophils,
eosinophils as well as dendritic cells (25, 26). This is associated with increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (27). ROS and RNS can produce tissue damage by reacting with various cell components resulting in DNA strand breaks, lipid peroxidation and protein oxidation. This chronic oxidative process can also lead to a progressive weakening of the antioxidant defense system. The overall increase in the production of ROS and RNS along with a reduction in antioxidants results in OxS (28).
I.A.3.1 Figure 1. Mechanisms of Airway Obliteration after Lung Transplantation

Reprinted with permission from Nicod (23)
I.A.3.2. Role of Ischemia/Reperfusion Injury (IRI)

Although the incidence of lung IRI has decreased from 30% to 15% (29) it significantly impacts early post lung transplant patient survival. IRI is characterized by non-specific alveolar damage, lung edema and hypoxemia (30) as a result of the lung graft’s dysfunctional pulmonary vascular endothelium, (31) representing the main source for the production of ROS and RNS in lung transplant recipients. There are several mechanisms by which IRI may increase OxS include: (a) increased production of hypoxanthine (32), (b) activation of the NADPH oxidase system (32), (c) increased iron release (33) and (d) activation of proinflammatory mediators.

OxS related to IRI has been studied in animal models where increased production of ROS/RNS was measured (34) and treatment with free radical scavengers such as antioxidant enzymes: superoxide dismutase and catalase was shown to be of benefit (35). These results suggest that antioxidants can reduce OxS in that setting. This was supported by another lung transplant animal model (36) where fourteen dogs received a continuous infusion of the free radical scavenger MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5) at the time of transplant. Results indicated a significant decrease in LPO measured by plasma MDA levels in the experimental group compared to the control group.
I.A.3.3 Role of Infections

Infections are also associated with OxS. Within the first year post transplantation, infections contribute to early mortality (37). Viral (including both Cytomegalovirus (CMV) and non-CMV), bacterial and fungal infections result in significant morbidity and mortality in lung recipients and are associated with the development of BOS (38,39).

Infectious episodes lead to the activation of inflammatory mediators resulting in increased production of ROS/RNS (OxS). This increased ROS/RNS production resulting from activated neutrophils, macrophages and monocytes, are essential for antimicrobial responses. A phagocytic process involving an intense consumption of oxygen is initiated and is known as the ‘respiratory burst’ (40). This process may lead to an excess production of free radicals including superoxide, hydrogen peroxide, peroxynitrite, hydroxyl radicals and singlet oxygen, all leading to lipid peroxidation, DNA and protein cellular damage (41). In addition, the resulting OxS can activate transcription factors such as nuclear transcription factor (NF-kB) and activator protein 1 (AP-1) (42) leading to a cascade of events in which pro-inflammatory cytokines {Interleukin 1-beta (IL-1β); TNF alpha (TNF-α)} and chemokines are released.

Therefore, infections are associated with OxS. For example, patients with Cystic Fibrosis (CF) have numerous infectious episodes
leading to increased OxS, and this has been measured by increases in plasma MDA (43) as well as plasma and urinary levels of 8-iso-PGF$_2$ iso prostanes (both end products of lipid peroxidation) (44).

Infections remain one of the leading causes of mortality within the first six months post lung transplantation, whereas BOS is the major cause of longer-term mortality.

**I.A.3.4 Role of acute rejection**

OxS has also been documented during acute rejection (45). Acute rejection is due to alloimmune-dependent factors that cause injury and inflammation to lung epithelial and endothelial cells (46). This inflammatory process is associated with the production of ROS/RNS leading to increased OxS (47).

Acute rejection is a significant contributing factor for the development of BOS and the number and severity of rejection episodes have been associated with its development (48-51).

Chemically active iron, released from ferritin stores as a result of tissue damage could add to the oxidative burden after lung transplantation. A cross sectional study, involving 14 stable lung recipients, 7 recipients with BOS and 21 normal controls, was conducted by Reid (52). They reported that there is microvascular leakage of iron within lung allografts and that this may contribute to OxS. Significantly elevated levels of ferritin (a storage form of iron,
which can act as a pro oxidant) and hemosiderin-laden macrophages in bronchoalveolar lavage fluid (BALF) were reported in both stable and BOS lung recipients when compared to controls (median 49 µg/L range 1-950 µg/L vs. 2 µg/L range 0-16 µg/L, p<0.01). The authors suggested that microvascular leakage in the lung allograft could potentiate iron overload, further exacerbating OxS and damage to the airway (52). This was confirmed in another study by Pugh (53), who reported significantly increased levels of BALF iron, transferrin and ferritin levels in transplant patients compared to healthy non-transplant control patients. Other factors such as altered antioxidant GSH levels during acute rejection episodes can also increase lung injury.

Lung recipients with acute rejection appear to have elevated levels of antioxidants when compared to those with no rejection; however, these levels were still lower than non-transplant healthy controls (45). Baz (45) analyzed 36 bronchoalveolar lavage fluid (BALF) samples from lung recipients who were approximately six-months post transplant to determine their GSH levels. Twenty-two lung recipients with no rejection and 14 patients with grade 2 rejection were compared to six healthy controls. Results indicated that healthy controls had the highest levels of total GSH (302.6 ± 40.8 µM) compared to patients with no rejection (94.0 ± 9.7 µM) (p<0.01).
Interestingly, patients with acute rejection had higher levels of GSH (179.8 ± 34.7uM) versus patients with no rejection (p<0.01). This is likely due to the increases in the oxidized portion of glutathione (GSSG), which occurs during inflammatory conditions such as acute rejection (45).

**I.A.3.5 Role of Gastroesophageal Reflux Disease (GERD)**

Lung transplant recipients with GERD have worsened pulmonary function (54). However, whether GERD may be an important contributor to the BOS pathogenesis is unknown. Pro-inflammatory factors, such as inflammatory cytokines (interleukin-6 and -8), leukocytes and OxS, have been demonstrated to be involved in the development of GERD (55).

**SUMMARY**

IRI, infections and acute rejection are major risk factors contributing to the development of BOS. In addition to the immunological reaction, these risk factors are also associated with increased OxS. One of the current hypotheses (56) regarding the pathogenesis of BOS is that chronic airway inflammation arises from rejection, possibly infection and repeated injury to the graft by ischemia-reperfusion. Chronic airway inflammation is associated with activated neutrophils, increasing the production of ROS/RNS, which produce OxS contributing to further tissue damage.
I.A.3.6 Role of OxS in Lung Transplant

OxS is a condition whereby prooxidants overwhelm the antioxidant defense system, and may contribute to the pathogenesis of BOS by inducing more tissue injury and inflammation.

Only two studies have assessed antioxidants in lung transplant recipients, and measured some OxS parameters. Williams (57) assessed lung recipients’ antioxidant status, both pre- and post-transplant by measuring levels of urate, ascorbate, thiols and α-tocopherol in both blood and bronchoalveolar lavage fluid (BALF) (57). Malondialdehyde (MDA), (an end product of lipid peroxidation) was also measured in both BALF and serum. Nineteen lung patients (with various lung diseases) were followed pre transplant then again at 2 weeks, 1,2,3,6, and 12 months post transplant. The group was also compared to 23 non-transplant healthy controls (HC). Pre-transplant patients had lower levels of serum thiol when compared to HC. Following transplant, serum levels of ascorbic acid, thiol and urate remained low, whereas MDA levels increased compared to HC. At 2 weeks post-transplant, BALF ascorbic acid levels remained low when compared to HC, BALF urate levels increased but there was no difference in GSH levels. BALF MDA levels were significantly elevated in transplant recipients’ vs HC, indicating that transplant recipients were oxidatively stressed. The authors concluded that the antioxidant
status of lung recipients was compromised pre-transplant and remained poor up to twelve months post transplant. These patients did not have BOS at the time of the study.

Another study performed in 15 CF patients taking vitamin supplements, including 8000IU (1200 µg) vitamin A and 300 IU (248 mg) vitamin E, assessed antioxidant vitamin levels (58). Serum vitamin A and vitamin E levels were determined pre-transplant and again at approximately 15 months post transplant. Pre-transplant serum vitamin A level was within the normal range 1.4 ± 0.5 µmol/L (reference range 0.7 to 2.1 µmol/L), while post-transplant, vitamin A serum levels significantly increased (3.1 ± 1.1 µmol/L). Similar results were seen regarding serum vitamin E levels. CF patients exhibited normal pre-transplant Vitamin E serum level (26.8 ± 8.9 µmol/L (reference range 12-46 µmol/L)), whereas the post-transplant level was significantly increased 44.2 ± 16.9 µmol/L. No measurements were conducted in BALF to assess lung antioxidant status or OxS. Supplementation with both Vitamin A and E (8000 IU, 300 IU respectively) remained the same pre-and post-transplant to prevent vitamin deficiencies. The increase in vitamin E and A serum vitamin levels post transplant may have been due to increased compliance or reduced OxS post transplant due to lower lung infectious rates related to CF.
In summary, only 2 studies were conducted examining OxS and lung transplantation. One study documented an increase in lipid peroxidation along with a weakened antioxidant status in lung transplants recipients not taking vitamin supplementation. The second study examining CF patients indicated that antioxidant vitamin supplementation helps maintains vitamin levels. No studies were performed assessing OxS in BOS.

**IA.4 BOS**

**IA.4.1 BOS and Inflammation**

Obliterative Bronchiolitis (OB) is difficult to diagnose; clinically, the term Bronchiolitis Obliterative Syndrome (BOS) is defined by changes in Forced Expired Ventilation in one minute (FEV₁). The staging system of BOS is outlined in IA.4.2 Table 1 (22).

BOS results in increased numbers of BALF-neutrophils being activated in the lung (59). Neutrophils are part of the lung innate immune system and with infection and/or rejection activated neutrophils (from the expression of pro-inflammatory cytokines such as IL-6 and-8 which attract these neutrophils) have a remarkable potential to cause damage to the lung tissue by generating ROS and RNS (21). Thus an increased number of neutrophils represents a marker of increased inflammation, and may be used as an indicator of OxS in lung recipients with BOS.
A number of studies have demonstrated that patients with BOS have increased neutrophil counts compared to patients without BOS (56, 60-64). One of the earliest studies examining Bronchoalveolar lavage fluid (BALF) in BOS patients was conducted by the Toronto Lung Transplant Group (60). The authors reported that BOS patients had a 31% mean neutrophil count whereas non-BOS patients had only 4%. In a case-control study, (62) examining 10 lung recipients with BOS (3 with BOS stage 3) and 9 non-BOS patients, results indicated that BOS patients who were 3 months post-transplant had both higher percentages of BALF neutrophils compared to non-BOS recipients. In another study, (64) comparing 24 lung recipients (74-287 days post transplant), 9 were free of BOS, and 5 had BOS. The 24 were compared to 18 HC. The results indicated that BOS patients had significantly higher levels of BALF neutrophils compared to both non-BOS and HC. These results confirmed previous studies (56) and (65). Thus, it appears that BOS lung recipients have increased BALF neutrophilia. This can lead to OxS and contribute to the development or progression of BOS, but further studies are needed to ascertain this link. Since BOS is resistant to most interventions, early identification may allow augmentation of immunosuppression with possible better outcomes (66). These increased neutrophil levels may also be useful in identifying BOS earlier.
An interesting prospective study provides convincing evidence that inflammatory markers may potentially be considered an early indicator of BOS. Digiovine (63) followed 59 lung transplant patients who had survived at least six-months post surgery. The purpose of this study was to ascertain the level and activity of BALF neutrophils, myeloperoxidase (MPO, a prooxidative enzyme) activity and Interleukin-8 (IL-8). Patients were divided into 2 groups, those with BOS (n=18) and those without BOS (n=41). Patients with BOS were further divided into ‘future BOS’ (representing those patients who later developed BOS) versus those as ‘BOS’ (patients with the diagnosis of BOS, at the time of biopsy). As well, those without BOS were divided into those with rejection (REJ) versus no rejection (Healthy). Not surprisingly, results indicated that patients with BOS had significantly higher levels of neutrophils, IL-8 and increased MPO activity compared to those without BOS either with or without rejection. Furthermore, those patients defined as ‘future BOS’ demonstrated a trend of increased neutrophil levels and IL-8 in BALF when compared to BALF concentrations in both the rejection and healthy groups, without BOS. Interestingly, the authors noted that these altered levels of neutrophils, increased IL-8 and MPO activity (all markers of inflammation that have been linked to OxS in other studies) occurred before decreases in lung function were noted. This study supports the
hypothesis that markers of inflammation leading to OxS may in fact predate the decline in FEV\textsubscript{1}. Thus, future prospective studies are needed to determine if OxS is involved in the pathogenesis of BOS, or if it is just a consequence of BOS.

**I.A.5 BOS and OxS**

As previously discussed, excess production of prooxidants can lead to OxS. As well, low levels of antioxidants can also tip the balance in favor of OxS (3). Several studies in BOS measured antioxidants but no one assessed direct parameters of OxS such as metabolites of lipid peroxidation.

Decreased antioxidant levels such as ascorbic acid, urate, α-tocopherol and GSH in both BALF and serum have been documented in BOS patients (67). Behr (67) measured BALF GSH levels in 19 non-BOS and 17 BOS patients, (3 with BOS Stage 1, 5-Stage 2 and 9 stage 3), over a 2-year period. The authors reported that GSH (Mean ± SEM) was significantly lower (162.6 ± 20.1 μM vs 345 ± 57.1 μM) in patients with BOS versus non-BOS. These results were further confirmed by Riise, (61) who compared 13 BOS post transplant patients with 13 non-BOS recipients. Results indicated that BALF antioxidant levels in BOS patients were lower compared to non-BOS recipients. Levels of ascorbic acid were 45% lower (0.31 (0-1.45) vs 0.56 (0.04-1.2) μmol/L), 13% lower for urate (2.64 (1.3-8.4) vs 3.04
(1.4-13.6 \mu\text{mol/L}), and 41\% lower for GSH \{(1.17 (0.36-5.47 vs 1.97 (59-4.97) \mu\text{mol/L}\}. As previously mentioned, under conditions of OxS GSH is rapidly converted to oxidized GSH (GSSG) and results from this study reported an 860\% increase in GSSG in BOS patients compared to non-BOS recipients. Taken together, these studies indicate that BOS recipients have lower antioxidant levels in BALF than non-BOS recipients; however studies on BOS recipients and direct markers of OxS such as lipid peroxidation (LPO) have not been conducted.

Current treatment for BOS is augmentation of immunosuppressive medications; however, this treatment often remains unsuccessful. A recent clinical trial on the use of azithromycin (AZA) (68) showed improvement in FEV\textsubscript{1} in BOS patients. AZA, is an antibiotic, that inhibits IL-8 release by human alveolar macrophages which in turn reduces OxS by decreasing inflammation. While this remains an experimental treatment it provides support for the contribution of OxS to BOS via inflammation.

In summary, lung recipients with BOS have increased activated neutrophils, which may be responsible for an increase in ROS production. This increase in ROS production with possible sub-optimal intakes of antioxidant nutrients may result in lowered levels of lung antioxidant defenses. This imbalance could enhance OxS, and contribute to the development and progression of BOS. However,
although low antioxidants in BALF were documented in BOS, OxS parameters such as metabolites from lipid peroxidation have not been reported in BOS lung recipients.

**I.A.6 FACTORS INFLUENCING OxS**

In addition to IRI and inflammation associated with rejection and infection, nutritional factors may contribute to OxS by either creating a pro oxidant status or affecting the antioxidant defense system. Prooxidant nutritional factors are obesity and dietary fat intake, particularly polyunsaturated fatty acids (PUFA). Conversely, nutritional factors contributing to the antioxidant defense systems are antioxidant micronutrients such as vitamins E, C and carotenoids. In addition antioxidant enzymes and trace elements like selenium also play a role.

**I.A.6.1 NUTRITIONAL FACTORS**

**I.A.6.1.1 Obesity**

We are currently encountering an obesity epidemic (69). Recent studies have indicated that decreased energy intake resulted in decreased OxS, measured by decreased plasma MDA levels in non-obese subjects compared to obese participants (70).

Obesity may be associated with a heightened state of inflammation (71) resulting in OxS. Increased body weight is positively associated with inflammation, measured by C-reactive protein levels (a
biomarker of inflammation) in obese individuals compared to non-obese (72,73).

Obesity is also prevalent in other solid organ transplant recipients (74-77). However, in the lung transplant population, there is minimal information on the association of obesity and the development of BOS.

Kanasky (78) examined 85 post lung transplant recipients (34% were overweight/obese) and determined that obesity had a negative effect on post transplant survival. The most powerful predictor of mortality was BMI, with an increased risk of death of 7% for each 1.0 unit (kg/m²) increase. These authors also reported that there was no difference in the development of BOS and/or infection in the obese compared to the non-obese group. Another study, also reported a negative impact of high pre-transplant BMI (79). Culver examined 46 lung transplant patients with BMI >30 kg/m² and reported a significant increase in 90-day mortality in obese lung transplant patients when compared to non-obese [OR 3.16 (CI:1.05-9.48)]. A retrospective chart review (80) of 826 lung transplant recipients from 12 international transplant centers reported that substantial weight gain occurred within the first year post transplant. In this study higher weight gain was associated with better subsequent survival (80).
Taken together, these studies indicate that there is a high prevalence of obesity post lung transplantation and that this may affect survival, although the results are mixed. Only one retrospective study looked at the relationship between obesity and BOS and did not find any increased risk of BOS in obese subjects (80).

I.A.6.1.2 Malnutrition

In addition to obesity, malnutrition can also be associated with OxS because of possible micronutrient deficiencies due to inadequate intake and/or malabsorption (81). In the pre-transplant lung literature the effect of malnutrition and OxS has been reported mainly in CF patients (82). CF patients have malabsorption of fat-soluble vitamins A, E and carotenoids (83,84). In addition, repeated or chronic infections as well as respiratory failure can increase catabolism and anoxeria contributing to malnutrition. Furthermore, chronic inflammation generate more ROS/RNS via neutrophil and macrophage activation and ‘respiratory burst’ (85,86). Therefore, the effect of reduced intake and increased malabsorption of micronutrient antioxidants, combined with greater demand of antioxidants for scavenging free radicals, will enhance OxS (85).

Several studies reported on malnutrition, malabsorption and low micronutrient levels pre-transplantation, particularly the CF population. In general, of the studies reported, OxS was shown to be elevated.
However, no studies have reported on nutritional status, dietary intake and plasma levels of antioxidant micronutrients in post lung transplantation particularly in BOS.

**I.A.6.2 Nutrition Intake**

**I.A.6.2.1 Polyunsaturated fatty acid (PUFA)**

The type of PUFA ingested in the diet can influence OxS and the demand for antioxidants. It can also influence underlying inflammation. Long chain PUFA are more prone to lipid peroxidation (LPO) because they have a greater number of double bonds. The greater the number of double bonds in the PUFAs, the more susceptible they are to LPO (87-89).

Hydroxyl radicals produced by activated neutrophils initiates this free radical chain reaction by attacking the double bonds of PUFA within the membrane phospholipids. Omega-6 PUFA such as arachidonic acid, and omega-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) are prone to LPO, particularly in an environment deficient in vitamin E. Both PUFA and vitamin E are influenced by diet.

In addition, the type of PUFA influences inflammation. Omega-3 PUFA such as α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) contained in walnuts, soybeans, flax seed oil and fish(90) have anti-inflammatory effects, by decreasing the
production of eicosanoids and leukotrienes, (91). On the other hand, omega-6 PUFA such as linoleic acid found in vegetable oils and arachidonic acid found in animal fat sources are classified as pro-inflammatory (92) as they produce two potent inflammatory mediators eicosanoids and leukotrienes (91). Therefore, the type of PUFA can influence OxS depending on its predisposition to LPO and its effect on inflammation. Both PUFA and vitamin E levels can be influenced by dietary intake.

The University of Toronto Lung Transplant Program conducts about 100-lung transplants/year, and patients with Cystic Fibrosis (CF) represent 23% of this population (UHN lung transplant data statistics; July 2009). These patients generally require high calorie and high fat nutrition care plan to optimize their nutritional status (93), along with pancreatic enzymes and vitamin supplementation to correct for malabsorption. In addition to the underlying disease, this regimen will influence OxS depending on the proportion and type of fat absorbed and the ratio of PUFA:vitamin E. High fat requirements lead to increased intake of PUFA, which lead to increased OxS.

Due to its anti-inflammatory effect, omega-3 PUFA is often given to CF patients as a supplement. Omega 3 supplements do contain vitamin E to prevent LPO. In a Cochrane systematic review (94) results indicated that CF supplemented with \( \omega-3 \) had improvements in
(a) lung function (b) improvement in clinical status (c) reduced volume of sputum and (d) increased essential fatty acids (efa) in neutrophil membranes. Few minor adverse effects were reported. Another study in CF patients found no change in OxS with fish oil supplementation (95), suggesting that vitamin E from supplementation was sufficient to prevent LPO. No $\omega$-3 PUFA studies have been conducted in the lung transplant population.

I.A.6.2.2 Antioxidants

Observational studies examining a possible protective role of antioxidants in lung diseases have been summarized in several review articles (96-98). Dietary studies form the bulk of these reports.

Current dietary guidelines from Health Canada *Eating Well with Canada’s Food Guide* recommend 7 food guide servings of fruit and vegetables per day (HC Pub:4651; Cat: H164-38/1-2007E SSBN:0-662-44467-1). Consuming foods based on this recommendation will provide a diet rich in antioxidants including vitamins C, E and $\beta$-carotene among others. Many dietary antioxidants work synergistically (99) to promote their protective effects as they exist in a natural environment and are biochemically balanced (100).

In the lung literature, diets rich in antioxidants have shown beneficial effects on lung function described as high maximal FEV$_1$. Cross-sectional studies have been conducted on 12,000 subjects with
various lung disease etiologies, indicating that intake of fruit and vegetable improves lung function (96-98,101). As well, large longitudinal studies have also reported a positive association between overall dietary intake of fruits and vegetables and FEV$_1$ (102-106). Furthermore, fruit and vegetable consumption has shown beneficial effects on decreasing respiratory symptoms and disease severity (107). Even low intake of fruit and vegetables compared to no consumption achieved a positive effect on lung function (96).

Similarly, one longitudinal study (102) reported an inverse association (RR=0.73) between fruit intake and the incidence of chronic nonspecific lung disease.

Studies on intake of fruits and vegetables and the effect on OxS measurements have been conducted. In a crossover study (108) increased consumption of 10 servings of fruits and vegetables resulted in a significant reduction of OxS measured as oxygen radical absorbance capacity (ORAC) from baseline to post consumption (108).

A dietary study examining the association between lung function, (described as high maximal FEV1) and intake of individual antioxidants provides conflicting results. Indeed, while some cross-sectional studies found a positive association between Vitamin C and lung function (109-112) along with two longitudinal studies (104, 105), others failed to find any association (102,113).
The evidence for intake of Vitamin E is also conflicting. Cross-sectional studies (109,110,113) and one longitudinal study (104) report a positive association between intake of vitamin E and lung function; whereas one cross-sectional (111) and one longitudinal study report no association (114).

Although less well studied, research indicates that intake of β-carotene shows similar trends. Two cross-sectional studies (111) (115) along with a longitudinal study (104) show a beneficial association whereas one cross-sectional (102) reports no benefit.

Studies involving patients with a variety of lung diseases have reported positive associations between plasma vitamin E, vitamin C and β-carotene antioxidant levels and FEV\textsubscript{1} (114,116).

Supplementation studies in patients with lung diseases have reported very disappointing results. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study (117,118) describes a RCT of 28,000 male smokers who were supplemented with daily dose of 50 IU of Vitamin E, 20 mg β-carotene, both or placebo for 5-8 years. Unexpected results indicated that vitamin E supplementation, increased the risk of death from hemorrhagic stroke, and β-carotene increased lung cancer mortality and ischemic heart disease.

The CARET study (119) tested effects of combined treatment of β-carotene (30 mg/d) and retinyl palmitate (25,000 IU/d) in 18,000
men and women with a history of smoking. Results from this study found increased risk of both lung cancer and coronary artery disease in treated patients compared to controls.

Therefore, most of these epidemiological studies showed some beneficial effect from antioxidants on various respiratory diseases. However, intervention studies were disappointing. Presently, minimal studies examining antioxidant status in lung transplant patients have been undertaken. One study (61) reported low BALF ascorbic acid and glutathione levels in BOS lung recipients when compared to non-BOS recipients. The second study (57) indicated elevated plasma and BALF MDA levels and low serum and BALF ascorbic acid up to 12 months after lung transplantation. No dietary intake assessment was performed and no intervention studies were conducted.

Given that BOS significantly reduces long-term survival and that its pathogenesis likely implicates OxS, it is of interest to assess OxS, antioxidants and nutrition in this patient population. In addition, depending on the results, the role of antioxidant supplementation in the lung transplant population may be considered in the future.

I.A.7 SUMMARY

Lung transplantation remains the main treatment for end-stage lung disease. While the one-year survival rate is excellent, the 5-year survival rate is poor, mainly due to BOS. IRI, infections, and rejection
are all associated with BOS. These complications can increase OxS and OxS may contribute to tissue damage and the pathogenesis of BOS.

While there are many studies on antioxidants and OxS in chronic lung disease pre-transplantation, there are very few studies evaluating OxS in lung recipients with BOS. More research is needed to assess OxS in this patient population and evaluate the role of nutrition and antioxidant micronutrients. This is what we sought in the first part of this research project.

**I.A.8 Specific Hypothesis and Aim**

The central hypothesis guiding the research project in this part of the thesis is the following

**Hypothesis:**
OxS will be increased in BOS lung recipients when compared to non-BOS patients. Furthermore, nutrients affecting OxS will be significantly different in BOS patients compared to non-BOS: i) higher PUFA and ii) lower antioxidant vitamins. This hypothesis was tested in a cross sectional study of 58 Lung transplant recipients.

**Aim:** To measure and compare OxS and nutritional intake in lung transplant patients with and without BOS.

**Summary of the Findings:**
Patients with more severe BOS (BOS 2-3) had significantly
o Higher Bronchoalveolar Lavage Fluid (BALF) LPO concentrations when compared to patients with milder stage of BOS (BOS Op-1) or with non-BOS (p=0.001, for both).

o Higher BALF oxidized glutathione (GSSG) concentrations when compared to patients with milder stage of BOS (BOS Op-1) (p=0.001) or with non-BOS (p=0.007).

However, BOS (2-3) patients did not have significant differences in

- BALF Antioxidant Potential (AOP) levels
- nutritional intake or
- plasma antioxidant levels

Overall, lung transplant patients had low intakes of beta-carotene and vitamin E compared to recommended intakes.
I.B: LIVER TRANSPLANTATION HCV DISEASE RECURRENCE AND OXIS

I.B.1 EPIDEMIOLOGY

Approximately, 170 million people worldwide are infected with Hepatitis C virus (HCV) (120), which was first described in 1990. This represents a global health problem reaching pandemic proportions (121). Canadian data indicate that ~250,000 to 300,000 Canadians are infected with HCV and this number is increasing.

The current therapy for HCV is Pegylated Interferon and Ribavirin (122,123). These medications are poorly tolerated with multiple side effects and in some circumstances patients may decide not to initiate therapy (124). In HCV genotype-1 patients, the sustained virological response (SVR) is only about 30-55% (125,126). The need to develop a more effective treatment has been identified and research studies are ongoing (127).

The natural history following HCV infection is characterized by chronic hepatitis, leading to the development of cirrhosis in approximately 30% of patients, followed by liver decompensation and ultimately the need for liver transplantation (128). HCV liver disease is the most common indication for liver transplantation worldwide (129).

In the United States there are 5,000 liver transplants performed per year and more than 16,000 patients are on the United Network for Organ Sharing (UNOS) wait list. In Canada, at the UHN liver
transplant program, 120 liver patients are transplanted yearly and 40% of these patients are infected with HCV (UHN database).

Unfortunately, post transplant, 100% of the recipients are reinfected with the virus (130) and 50% of the patients develop histological evidence of HCV disease recurrence by one year (131). Ten to thirty percent of the patients, progress to cirrhosis by 5-years post transplant, thus HCV-LT has lower survival rates than other non-HCV liver recipients (132).

**I.B.2 Pathogenesis of HCV**

HCV is a single-stranded enveloped positive-sense RNA virus of the family Flaviviridae. HCV genome comprises core, structural and non-structural proteins within the polyprotein (133). HCV is a genetically heterogeneous virus with the ability to mutate rapidly and lacks a protective antibody response. These two factors together pose a significant challenge to those scientists attempting to develop an HCV vaccine (134).

Viral replication involves a complex process. The virus enters a hepatocyte, and after entry, the virus ‘uncoats’ and undergoes a translational process within the endoplasmic reticulum (ER)-derived membranous web (135), resulting in increased viral replication. HCV replication disrupts normal ER functions and induces ER-stress. This then results in a disruption to the normal calcium signaling process.
occurring in the ER and causes increased production of ROS/RNS (136).

In an attempt to diminish the invading pathogen, the human body initiates an immune response, primed by macrophages and dendritic cells that present viral proteins to B cells, helper T cells and cytotoxic T cells (137-142). Chronic HCV infection leads to chronic inflammation, resulting in increased production of ROS/RNS, DNA damage and apoptosis (143).

OxS has been shown to contribute in the pathogenesis of several liver diseases such as non-alcoholic steatohepatitis (NASH) (144) alcoholic liver disease (145) and other liver disorders such as Wilson’s disease (146). However for the purpose of this project we will focus on HCV.

OxS can be produced from HCV, and it may also contribute to HCV viral replication, although the exact mechanism is not fully delineated. One proposed mechanism is that HCV proteins deplete Ca\(^{2+}\) stores in endoplasmic reticulum which combined with increased Ca\(^{2+}\) uptake by mitochondria leads to mitochondrial injury and increased mitochondrial production of ROS/RNS (147,148). ROS/RNS can then act as secondary messengers and activate NF\(\kappa\)B (149), leading to increased HCV replication (150). This is a similar mechanism as observed in HIV infection where ROS can enhance HIV
replication by activating NFκB (151,152). On the other hand, one in vitro study has reported that ROS may suppress HCV RNA replication (153) by disrupting active HCV replication complexes. Therefore it is not clear whether OxS contributes to HCV RNA replication.

I.B.3 Pathogenesis of HCV and OxS

The liver is the most metabolically active organ in the body, performing multiple interrelated functions that are essential for life (154). Many of these functions are associated with the production of ROS/RNS, which are immediately scavenged by the antioxidant defense system.

The main source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in hepatocytes is the mitochondria. ROS are also produced outside hepatocytes, from NADPH oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. Although the majority of oxygen is utilized for normal aerobic processes, approximately 2-3% of consumed oxygen is constantly converted into ROS/RNS in the mitochondria accompanied by oxygen consumption in the electron transport system (155). Hepatocytes contain many mitochondria and therefore generate excess ROS/RNS. These ROS/RNS are unstable and highly reactive, attacking biomolecules such as DNA, lipids, and proteins (156). To prevent this damage the liver has a very
sophisticated antioxidant defense system, which involves micronutrients and enzymes (155).

The pathogenesis of Chronic Hepatitis C (CHC) infection is not completely understood, but several studies have suggested that there are at least two mechanisms involved in the production of ROS/RNS: one is from the core protein itself (155) and the other is from the inflammatory process (157).

In addition, another contributing factor that may increase OxS in the context of HCV infection is hepatic steatosis (158) either induced by HCV genotype 3 or insulin resistance. Increased production of ROS/RNS from HCV infection can also affect antioxidant enzyme activity contributing to OxS.

Several studies (165,168,172) investigated the effect of HCV on the antioxidant system and the results are conflicting. Although HCV infection causes increased OxS (159), the mechanisms of this altered oxidant:antioxidant imbalance from the core proteins or non-structural protein expression are different (160). One in vitro study using hepatocyte cell lines and an oxidant-sensitive probe 5-(and-6)-chloromethyl- 20,70-ichlorodihydrofluorescein diacetate (CMDCFH2) fluorescence (which measure OxS), reported that core protein expression resulted in decreased glutathione (GSH), and an increased oxidation of thioredoxin (Trx), a stress inducible enzyme. In
addition, the expression of HCV-non-structural (HCV-NS) proteins also led to an increase in OxS, but the responses from the antioxidant enzymes were different. Increases in antioxidant enzyme activity MnSOD and catalase, heme oxygenase-1 (HO-1), and an increase in GSH, were reported. Thus, patients with CHC are oxidatively stressed, and different viral proteins express different mechanism(s) of prooxidative activity (160).

**I.B.4 OxS AND ANTIOXIDANTS IN NON‐TRANSPLANT POPULATION**

Many studies assessed various parameters of OxS and antioxidant defense system in humans with CHC. Most of these studies demonstrated an association between CHC infection and either an increase in OxS or a decrease in antioxidants. However, several of these studies have limitations and most were performed in the non-transplant population. While some studies focused on antioxidants, others measured both OxS parameters and antioxidants or OxS alone. A large proportion of the studies are observational and most are cross sectional, they are summarized in the following table. Increased liver iron concentrations also promote increased OxS (161) and this has been previously documented in non-transplant HCV patients (162).
### I.B.4. TABLE 1. OxS and Antioxidant Studies in CHC patients

<table>
<thead>
<tr>
<th>Author/Reference</th>
<th>N</th>
<th>Study Design</th>
<th>OxS Marker</th>
<th>Antioxidant</th>
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<td>CC</td>
<td>↑ serum TRX</td>
<td></td>
</tr>
<tr>
<td>Barbaro (164)</td>
<td>130 CHC</td>
<td>CS</td>
<td>↓ Hepatic GSH; ↓ serum GSH</td>
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<td>↓ Serum GSH</td>
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<td>CC</td>
<td>↑ serum MDA; ↑ serum PCC</td>
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<td>CS</td>
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<td></td>
</tr>
<tr>
<td>Jain (168)</td>
<td>42 CHC</td>
<td>CC</td>
<td>↑ urinary 8-isoprostane</td>
<td>↑ Plasma GSH:GSSG; ↓ GSH; ↓ Sel; ↓ Vit A; ↓ Vit C; ↓ Vit E</td>
</tr>
<tr>
<td>Farinati (169)</td>
<td>42 CHC</td>
<td>CS</td>
<td>↑ hepatic MDA</td>
<td>↑ hepatic GSH; ↑ hepatic GSSG</td>
</tr>
<tr>
<td>Larrea (170)</td>
<td>44 CHC</td>
<td></td>
<td></td>
<td>↑ Mn-SOD</td>
</tr>
<tr>
<td>Paradis (171)</td>
<td>43 CHC</td>
<td>CS</td>
<td>↑ hepatic MDA</td>
<td></td>
</tr>
<tr>
<td>Gronbaek (172)</td>
<td>23 CHC</td>
<td>CS</td>
<td></td>
<td>↓ Plasma β-Carotene; retinol; AA; α-toco</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- **CHC**: patients with Chronic hepatitis C infection; **TRX**: thioredoxin; **CAT**: Catalase; **SOD**: superoxide dismutase; **GPx**: glutathione peroxidase; **GSH**: glutathione; **GSSG**: oxidized glutathione; **PCC**: protein carbonyl content; **MDA**: malondialdehyde; **Sel**: selenium; **HC**: healthy controls; **CC**: Case Control; **CS**: Cross Sectional; **Mn-SOD**: Manganese-superoxide dismutase; **AA**: ascorbic acid; **α-toco**: alpha-tocopherol
Increased activity of the ROS scavenging system including decreased hepatic and blood glutathione (GSH) levels, along with increased oxidized glutathione/glutathione ratio has been reported in patients with CHC (164,169). Other authors also reported increased levels of serum thioredoxin (TRX) indicating that HCV patients are oxidatively stressed (163). Similarly, increased activity of enzymatic antioxidants: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) have also been reported in HCV patients (160), suggesting enzymatic induction from chronic OxS.

In Bandara’s study (165) the relationship between plasma antioxidants and liver histology was studied. Glutathione and vitamin C, but not vitamin E correlated with both portal/periportal activity ($r=-0.19, p=0.004; r=-0.19, p=0.009$ respectively) and fibrosis stage ($r=-0.18, p=0.007; r=-0.18, p=0.009$ respectively). Antioxidants, glutathione and vitamin C were both independent negative predictors of portal/periportal inflammation ($p=0.02$) and fibrosis ($P=0.01$), whereas only vitamin C was an independent negative predictor of fibrosis stage ($p=0.02$).

In a comparable study, DeMaria (166) reported that patients with CHC had higher serum levels of malondialdehyde (MDA), protein carbonyl content (PCC) and reduced levels of glutathione, all indicators of OxS, when compared to HC. Both serum MDA and protein carbonyl
content correlated with serum ALT levels ($r = .792$ and $r = .818$ respectively, $p < .001$). Although iron may contribute to the elevation of OxS and increase risk of liver damage, no correlation was found between MDA or PCC and the hepatic iron content. The authors concluded that: (i) lipid and protein oxidation occur in CHC, (ii) oxidative damage can be demonstrated by increased serum levels of MDA and PCC, and (iii) both MDA and PCC levels correlate with disease activity (166).

As in the DeMarias study, increased plasma MDA in patients with CHC and low levels of antioxidant enzymes Cu and Zn SOD and glutathione peroxidase were seen when compared to HC (173).

In another study Jain (168) reported that both plasma levels of 8-isoprostane (an LPO marker) and the ratio of oxidized to reduced GSH levels were significantly elevated in patients with CHC. In a subgroup analysis comparing CHC cirrhotic and non-cirrhotic patients with HC, both groups had significantly decreased levels of antioxidants: glutathione, selenium and vitamins A, C and E (all $P<0.001$). As well, although cirrhotic patients had more markedly abnormal values; significant changes were also seen in non-cirrhotic patients. Fibrosis score positively correlated with urinary 8-isoprostane (a LPO marker) and type III procollagen peptide, while vitamin A was negatively correlated with fibrosis score. The authors
note that a significant feature of CHC infection is OxS and this may suggest a role for antioxidant supplementation (168).

Yadav (174), reported that patients with CHC had significantly higher serum MDA levels compared with HC (1.62 ± 0.57 versus 0.23 ± 0.15 µmol/L). As well, most plasma micronutrient antioxidants: retinol, alpha and gamma-tocopherol and β-carotene were two to ten times lower in patients with CHC compared to HC. In addition, patients with CHC with moderate-to-severe inflammation or fibrosis had significantly higher serum MDA levels and significantly lower serum antioxidants when compared to those with mild inflammation or fibrosis. However, hepatic levels of the antioxidants retinol, alpha and gamma-tocopherol, and β-carotene were not associated with the severity of inflammation (portal or lobular).

In the non-transplant CHC population, an association between OxS or decreased micronutrient antioxidant serum levels and HCV-RNA levels has been reported (175,176). In Ko’s study (175) results indicated that antioxidant micronutrients (zinc, selenium, copper), were lower, particularly selenium, and several indicators of OxS such as serum MDA were elevated in CHC patients when compared to HC. As well, correlations between antioxidants and OxS and viral load in CHC patients were seen (175).
Likewise, Gronbaek (176) reported a possible association between HCV load and antioxidant status. Results indicated that the higher the viral load, the lower the antioxidant levels, based on comparison of antioxidant levels for each log increase in viral load. Plasma retinol, ascorbic acid and α-tocopherol were low in 17%, 26% and 4% of the patients, respectively. Plasma ascorbic acid and α-tocopherol was lowered by 9.7 mmol/l (95% CI 3.3-16.2) and 4.5 mmol/l (95% CI 2.1-7.0) respectively, and plasma β-carotene decreased by a factor of 0.60 (95% CI 0.37-0.98) per log increase in viral load. This suggests a possible relationship whereby increased viral load may increase OxS and thus consumption of antioxidants.

Therefore, these studies support an association between OxS and HCV. However, the majority of studies were cross-sectional or case-control. There were no prospective cohort studies looking at OxS as a potential contributing factor to disease progression. However, considering the significant association between OxS and HCV, some studies on HCV related liver disease have examined the effect of antioxidant supplementation.

**I.B.5 Antioxidant Supplementation Intervention Studies**

Results of human intervention studies on antioxidant supplementation in patients with CHC have reported mixed results. The following is a summary of those studies (I.B.5 Table 1).
### Table 1. Antioxidant supplementation studies in CHC patients

<table>
<thead>
<tr>
<th>Author reference</th>
<th>N</th>
<th>Study Design</th>
<th>Antioxidant</th>
<th>Study duration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ota(177)</td>
<td>8 HCV 8 HC</td>
<td>CC</td>
<td>500 mg Vit E</td>
<td>3 M</td>
<td>↓ ALT, ↓ Plasma t-bars</td>
</tr>
<tr>
<td>Murkami(178)</td>
<td>14 Supp 16 no supp</td>
<td>RCT</td>
<td>IFN +500 mg Vit E +750 mg Vit C IFN no supplement</td>
<td>2 M</td>
<td>↓ ALT No Δ EPA ↑ Plasma Vit C E</td>
</tr>
<tr>
<td>Groenbaek (179)</td>
<td>23</td>
<td>RCT</td>
<td>500 mg Vit C 945 IU Vit E 200 µg Sel</td>
<td>6 M</td>
<td>No Δ ALT, or plasma MDA ↑ Plasma Vit C E ↑ GPx No Δ viral load</td>
</tr>
<tr>
<td>Emerit(180)</td>
<td>15 15</td>
<td>RCT</td>
<td>Phenol rich supplement</td>
<td>3 M</td>
<td>↓ AST ↑ AO levels No Δ viral load</td>
</tr>
<tr>
<td>Ideo(125)</td>
<td>120</td>
<td>RCT</td>
<td>IFN +1200 mg NAC +600 mg Vit E vs IFN alone</td>
<td>6 M</td>
<td>No Δ viral load</td>
</tr>
<tr>
<td>Look(181)</td>
<td>24 3 grps</td>
<td>RCT</td>
<td>IFN IFN+NAC (1800 mg) +Sel (400µg) IFN+NAC+Sel+Vit E (500 mg)</td>
<td>3M</td>
<td>Vit E grp only: 2.4X &gt; SVR ↓ viral load</td>
</tr>
<tr>
<td>Melhem(182)</td>
<td>50</td>
<td>Clinical Trial</td>
<td>AO mixture</td>
<td>6M</td>
<td>↓ viral load ↓ ALT;HAI</td>
</tr>
<tr>
<td>VonHerbay (183)</td>
<td>23</td>
<td>RCT</td>
<td>800 IU Vit E</td>
<td>3M</td>
<td>↓ ALT ↑ Plasma Vit E</td>
</tr>
</tbody>
</table>

**Abbreviations:** CC: case control; AO: antioxidant; ALT: IFN: Interferon-2α; SVR: sustained virological response; RCT: randomized controlled trial; GPx: glutathione peroxidase; EPA: eicosapentaenoic acid; NAC: N-acetyl cysteine; Sel: selenium; CRP: C-reactive protein; AO mixture: glycyrrhizin, schisandra, silymarin, ascorbic acid, lipoic acid, L-glutathione, and alpha-tocopherol for 20 weeks, along with four different intravenous preparations (glycyrrhizin, ascorbic acid, L-glutathione, B-complex) twice weekly for the first 10 weeks, and followed up for an additional 20 weeks.
Improvements in ALT were reported with (a) supplementation of Vitamin E (177); (b) Vitamin E in combination with Vitamin C (178) and (c) phenol-rich supplement (180). Some studies have reported no change in viral load with (a) Vitamin C plus Vitamin E and Selenium (179), (b) phenol-rich supplement (180) and (c) N-acetyl-cysteine (NAC) combined with Vitamin E (125). In contrast to this, another supplementation study with a mixture of antioxidants given for 20 weeks, along with four different antioxidant intravenous preparations given twice weekly for the first 10 weeks, and followed up for an additional 20 weeks indicated a 2-point reduction in Histological Activity Index (HAI) (182). Similarly, decreased viral load was reported in patients with CHC taking supplementation of NAC along with both Selenium and Vitamin E (181). However, the largest and longest RCT examining vitamin E as adjunctive therapy with IFN reported no change in viral load (125).

Therefore, several studies have documented the presence of OxS in patients with CHC and investigated the effect of antioxidants. The study results are conflicting due to different study endpoints, different amounts and types of antioxidants and duration of the study. Some of these studies have small sample size and overall, adding antioxidants to interferon may not provide an additional significant benefit. However, some of these patient populations are different:
greater benefit from antioxidants may be detected in those who are at risk of antioxidant deficiencies, such as those with a history of drug addiction or HIV as compared to those who were infected via blood transfusion or sexual transmission. It is not known whether those who were low on antioxidant at baseline had a better response. Considering that liver transplant recipients can be oxidatively stressed with low antioxidants, it would be of interest to further explore the role of antioxidant supplementation, in this population, once we have examined the role of OxS in HCV disease recurrence.

**I.B.6 OxS and Liver Transplantation**

We have previously determined that OxS occurs in lung transplant recipients (Tx Proceedings, Nov 2009, in press). As well other authors have reported increased OxS in heart recipients and renal recipients (184-186). In HCV liver recipients, no research has been reported on hepatic LPO in the context of HCV disease recurrence. Most of the research on OxS in the post transplantation setting has been conducted specifically to examine the role of Ischemia- Reperfusion Injury (156).

**I.B.6.1 OxS and Ischemia-reperfusion Injury (IRI)**

Patients with end stage liver disease are oxidatively stressed due to ongoing liver disease and low antioxidant micronutrients. Following transplantation, IRI can also produce OxS.
The mechanism of increased ROS/RNS in IRI is similar to the mechanism seen in lung transplant recipients and is related to the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase, which generates the superoxide radical via univalent reduction of O$_2$ in the final catabolic route of ATP and ADP (187). There is some evidence to suggest that IRI represents a more significant problem in cadaveric as compared to living-related transplants (188,189) but this is not been conclusively confirmed (190).

The most important source of liver graft damage is the overproduction of oxygen reactive species (ROS) during the initial phase of reperfusion, resulting in widespread damage to proteins, lipids, and DNA (156). To maintain cell integrity these effects are counterbalanced by efficient antioxidant mechanisms, including glutathione (GSH).

Hassan (191) prospectively followed 13 liver transplant patients evaluating the temporal variations of antioxidant enzyme activities in liver transplant recipients. Blood samples were obtained pre-and post transplant (1, 6, 12, 24, 48,72 hours), and again at 5 and 7 days post transplant. Antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) were measured. OxS was measured by determining...
plasma malondialdehyde (MDA) levels. Results indicated that SOD and GPx activities were elevated post transplant, and these levels correlated with plasma MDA levels. The Manganese-SOD (Mn-SOD) (75%) appeared to be the major SOD activity compared to Cu and Zn-SOD (25%). In contrast to this, the Fe-SOD component was not detected. These authors concluded that increased OxS occurring post transplant likely induced antioxidant enzyme activities. Furthermore, although MDA levels increased enormously by 1 hour after transplant, this increased lipid peroxidation was compensated for by increased GPx activity (191).

Antioxidant supplementation to prevent and/or minimize hepatocellular damage due to IRI has been proposed by Grezzana (156) and colleagues and the beneficial role of antioxidant supplementation to diminish OxS in the early post-operative period has been reported.

A number of animal studies have been conducted indicating that lipid peroxidation can be reduced in the setting of IRI with supplementation of ascorbic acid (192); NAC (193) and SOD derivatives (194).

Other authors have also investigated the use of antioxidants to help ameliorate the effects of post-transplant IRI (195-199).
**I.B.7 Evidence of OxS in Long-term Liver Transplants**

Trevisani (200) and Burke (201) showed that OxS could be elevated in longer-term liver transplant recipients.

Burke (201) reported that OxS, based on urinary measurements, was evident in post transplant recipients 1 year after surgery. They prospectively followed 50 pre and post liver transplant patients: 25 HCV negative and 25 HCV positive and compared them to 30 healthy controls (HC). OxS was measured by determining levels of urinary dinor-dihydro iP(2α-111) levels. These results indicated that increased OxS occurred in: pre-transplant patients versus controls; early post transplant patients versus pre-transplant; and pre-transplant versus 12 months post liver transplant. However, there was no significant difference in OxS measurements in patients with HCV disease recurrence (n=15) compared to those with no recurrence (n=10). This non-significant finding could be related to the low sample size or the choice of OxS measurement. As well, no associations between OxS and acute cellular rejection, organ failure, or infection of the allograft by HCV were noted, suggesting that the marker of OxS measurement may not have been appropriate. While this OxS marker (urinary dinor-dihydro iP(2α-111)) is highly reproducible, sensitive, and specific, it is however very labour intensive, and is not used in our
laboratory. It is also not necessarily reflecting the level of OxS in the liver.

Trevisani (200) investigated the relationship between plasma OxS markers and HCV-LT in two studies. The first was a cross sectional study reporting on 20 pre transplant cirrhotic patients and 22 post LT patients (who were at least six- months post transplant) and 30 HC. The second part was a longitudinal study consisting of 5 liver transplant patients who were followed before and again between 6-12 months post transplant. OxS was measured by examining plasma concentrations of thiobarbituric acid-reactant substances (TBARS) and serum antioxidant levels of $\alpha$-tocopherol pre and post transplant. Not surprisingly, pre-transplant liver cirrhotic patients had increased OxS, represented by elevated TBARS with corresponding depressed $\alpha$-tocopherol levels when compared to post transplant patients and controls ($p=0.043$). Following transplant, there was a significant decrease in TBARS levels with an increase in $\alpha$-tocopherol levels. However, the levels did not normalize when compared to HC. The longitudinal component of the study indicated that all five liver recipients demonstrated improvements in OxS measurements and antioxidant levels but these values did not normalize when compared to controls. No correlations were seen between OxS parameters and liver function tests, disease recurrence or rejection episodes. These
two studies indicate that mild OxS persists even in successfully transplanted patients.

*To summarize,* a significant amount of literature indicates that pre-transplant liver patients are oxidatively stressed. Several animal studies also report enhanced OxS immediately following transplant, possibly due to IRI. Although there is some improvement of OxS post transplant it still remains higher than normal after six months. However, hepatic OxS and its potential association with HCV disease recurrence have not been adequately examined.

**I.B.8 Factors influencing Disease Recurrence post transplant**

While every attempt is made to eradicate the virus pre-transplant the development of HCV-related cirrhosis post liver transplant remains a significant ongoing problem. Established risk factors associated with the progression of HCV disease recurrence include: pre-transplant viral load, early post-transplant viral load, steroid bolus for acute rejection episodes, CMV infection and donor age (202-206). OxS may be another risk factor.

**I.B.9 Factors influencing OxS and HCV**

A number of factors have been identified that are associated with OxS, including medications, smoking and nutritional factors.
I.B.9.1 Medications

Cyclosporine A (CsA) and Tacrolimus (TAC) are potent immunosuppressive agents used for the prevention of graft rejection. Both drugs are metabolized through the cytochrome P450 system and are associated with intracellular depletion of reduced glutathione and increased lipid peroxidation and possess pro-oxidant activity (207). CsA has been shown to increase the production of ROS in rat microsomes (208), and in human microsomes (209) leading to increased LPO and this was confirmed in another animal study (210). TAC also increases the production of ROS (211).

I.B.9.2 Smoking

Cigarette smoke a well known environmental source of free radicals resulting in oxidative cellular damage (212, 213) lipid peroxidation (214).

I.B.9.3 Infections

Infections can lead to increased inflammation and enhanced generation of ROS/RNS via the ‘respiratory burst’ of neutrophils and macrophages (85,86).

I.B.9.4 Alcohol

Approximately 80% of the ingested ethanol is metabolized in the liver leading to excess generation of ROS/RNS, triggering the inflammatory response (215,216).
I.B.9.5 NUTRITIONAL FACTORS

Nutritional factors influencing OxS were already discussed in Section II.A.1 under lung transplantation and will only be briefly mentioned here.

I.B.9.5.1 Intakes

I.B.9.5.1.1 Polyunsaturated fatty acids

The greater the number of double bonds in the polyunsaturated fatty acids, the more susceptible they are to oxidation (87-89). The OH• radical from neutrophil and macrophage activation can initiate the free radical chain reaction known as lipid peroxidation by attacking the fatty acid side chains of membrane phospholipids, leading to tissue damage.

I.B.9.5.1.2 Antioxidant intake

Poor intakes of micronutrients including vitamins A, E and C all contribute to a poor antioxidant defense system (217). Plasma levels of antioxidants such as Vitamin E, C and β-carotene all affect LPO indices.

I.B.9.5.2 NUTRITIONAL STATUS

I.B.5.2.1 Malnutrition

Patients’ nutritional status also plays a role in OxS. Malnourished patients have increased OxS due to inadequate micronutrient intakes and/or malabsorption (218).
**I.B.9.5.2.2 Obesity**

The development of OxS could be influenced by obesity because increased body weight is positively associated with inflammation (72). Studies have also shown that transplant patients with BMI > 30 kg/m², have increased risk of mortality and graft loss (219). Results from the United Network for Organ Sharing (UNOS) database have indicated that both high and low BMI was predictive of death-censored graft loss (219).

Obesity, insulin resistance (IR), glucose intolerance, hypertension and dyslipidemia associated with metabolic syndrome and NAFLD can increase OxS and a significant number of transplant patients exhibit this syndrome (220). A large liver transplant study examining graft and patient survival in 8,382 liver recipients reported 46% of the patients were non-obese; 33% overweight; 14% obese; and 5% were classified as severely obese indicating a total of 54% of liver recipients as either overweight or obese (221). In a case control study of 121 liver recipients Nair (222) reported that obese liver recipients had increased respiratory failure; systemic vascular complications; peri-operative complications; longer length of stay and increased hospital costs when compared to non-obese. However, long-term survival was similar in both groups. In a review of 1300 liver recipients from prospective studies, results indicated that 5-year
survival rates for patient or graft did not differ with increasing obesity (223). Therefore, most studies suggest that obesity can affect clinical outcomes. It is also associated with the metabolic syndrome post transplantation (224-226). This can lead to NAFLD, which is associated with OxS (227).

**I.B.9.6 Other factors**

Exercise has been shown to cause increased OxS (228, 229). Increased oxidative phosphorylation with concomitant increase in the production of free radicals occurs with exercise (230) leading to LPO, if ROS/RNS exceed antioxidants.

**I.B.10 Summary**

HCV is a major cause of hepatic cirrhosis, and is the leading indication for liver transplantation. It has been demonstrated that HCV liver patients are oxidatively stressed pre-transplant that IRI may contribute to early OxS post-transplant and that, based on plasma or urine measurements, post transplant patients remain with some degree of OxS in the long term. However, studies on hepatic OxS and its relation to HCV disease recurrence, as well as potential contribution from nutritional factors have never been conducted.

One hundred percent of HCV-LT are re-infected with the virus as early as 2-3 weeks post transplant and 50-60% develop HCV-induced graft fibrosis within one year post transplant. It has also been
documented that liver recipients with recurrence, have poorer prognosis and earlier complications then immunocompetent non-transplant HCV patients (231). Unfortunately, HCV disease recurrence is inevitable and highly unpredictable. Thus research to minimize the consequences of disease recurrence and its impact on graft function remain important. Previous research has not addressed whether or not OxS as reflected by hepatic LPO is present in HCV-LT at six-months post transplant. Nor has it determined if those patients with recurrence are more oxidatively stressed at 12 months post transplant compared to those with no recurrence. In addition, it is not known if OxS is associated with HCV disease recurrence.

Therefore, investigating OxS and nutritional factors in HCV liver transplant recipients’ merits exploration by assessing hepatic lipid peroxidation at six- and 12 months post transplantation and addressing the following questions:

1. At six-months post transplant do HCV-LT remain OxS?
2. Is OxS increased in HCV-LT with recurrence compared to no recurrence at 12 months?
3. Is OxS associated with HCV disease recurrence?
The next portion of this thesis will address these questions in three separate studies.

**I.B.11 SPECIFIC HYPOTHESES AND AIDS**

**Hypothesis 1:**
HCV-LT at six-months post transplant, without disease recurrence, are more oxidatively stressed compared to Controls and HCV non-transplant patients. This hypothesis was addressed in a cross sectional study of 33 HCV-LT; 12 Controls and 39 HCV-non-transplant patients.

**Aim 1:**
To determine if HCV-LT with no disease recurrence at six-months post transplant had:

1. Increased Liver LPO;
2. Decreased liver and plasma AOP;
3. Decreased Plasma antioxidants;
4. Lower antioxidant intake

**compared to** HCV non-transplant and Controls who were hepatology patients assessed for mildly increased liver enzymes and found to have normal investigations including normal or minimal findings on liver biopsy.

**Summary of the findings:**
➢ HCV-LT with no disease recurrence have higher hepatic LPO when compared to both Controls and HCV non-transplant patients
➢ Plasma Antioxidant Potential (AOP) was significantly lower in HCV-LT when compared to Controls
➢ Plasma AOP was significantly lower in HCV-NT when compared to Controls
➢ Plasma gamma-tocopherol was significantly higher in HCV-LT when compared to Controls
➢ HCV-LT consumed significantly less Vitamin A (µg/d) compared to both Controls and HCV-NT
➢ Waist-to-Hip Ratio (WHR) was significantly higher in HCV-LT when compared to Controls

**Hypothesis 2:**

HCV-LT at 12 months post transplant with HCV disease recurrence are more oxidatively stressed compared to those HCV-LT with no HCV disease recurrence.

**Aim 2:**

To determine whether patients with HCV disease recurrence at 12 Months post transplant have:

1. Increased Liver LPO;
2. Decreased liver and plasma AOP;
3. Decreased Plasma antioxidants;

4. Lower antioxidant intake

**compared to** those HCV-LT with no disease recurrence.

**Summary of the Findings**

- At 12 months, post liver transplantation, HCV-LT with disease recurrence have higher hepatic LPO levels indicating that these patients are more oxidatively stressed when compared to those HCV-LT with no disease recurrence.

**No differences were seen in the two groups between**

- Liver AOP;
- Plasma AOP;
- Plasma Antioxidants, Vitamin C, Retinol, gamma or alpha-tocopherol;
- Intakes of macro and micronutrients

**Hypothesis 3:**

HCV-LT with recurrence at 12 months were more oxidatively stressed at six-months, when compared to HCV-LT with no recurrence at 12 months.

**Aim 3:**

To determine if HCV-LT with disease recurrence at 12 months had

1. Higher levels of liver LPO;
2. Lower levels of Liver and plasma AOP;
3. Lower levels plasma antioxidants;

**4. Lower intakes of antioxidant micronutrients**

*at six-months*, when compared to those with no disease recurrence.

**Summary of the findings:**

- HCV-LT with disease recurrence at 12 months, had higher hepatic LPO levels at six-months when compared to those with no recurrence
- No differences were seen between the two groups for the other parameters
- Due to a small sample size for Aim 3, results are provisional

**INNOVATION IN THE FIELD OF TRANSPLANTATION**

- These lung and liver studies have provided evidence that transplant patients are oxidatively stressed.
- BOS lung recipients had higher BALF levels of LPO and GSSG (both indicators of OxS) compared to non-BOS lung recipients.
- This is the first report of HCV-LT with higher hepatic LPO levels when compared to Controls and HCV-NT.
- This is the first documentation that HCV-LT at 12 months post transplant with HCV disease recurrence had higher
hepatic LPO levels when compared to HCV-LT with no recurrence.

- Although provisional due to the small sample size, this is the first prospective study to examine hepatic LPO in HCV-LT at six and 12 months post transplant.
- HCV-LT with recurrence at 12 months post transplant had higher hepatic LPO levels at six-month post transplant when compared to HCV-LT with no recurrence.
  - This is a novel finding since liver histology indicated no difference in necroinflammatory activity at six-months post transplant in HCV-LT with recurrence compared to HCV-LT with no recurrence.
- The potential of an OxS biomarker to help determine which lung recipients will develop BOS or which HCV-LT will develop disease recurrence warrants further evaluation.
- Findings from these transplant studies may support future pilot studies looking at the effect of antioxidant dietary interventions and/or antioxidant micronutrient supplementation on OxS and i) pulmonary function in lung recipients and/or ii) HCV disease recurrence in liver recipients.
CHAPTER II: MATERIAL AND METHODS

This section concentrates on the methodological approach used to assess OxS, antioxidants and nutritional status in both parts of the thesis: OxS in lung recipients and OxS in HCV-LT. This will be followed in the next three chapters, by the published or submitted article of each study, describing the design, results and discussion.

II.A.1 LUNG TRANSPLANTATION, FEV₁ AND BOS STAGING

BOS is defined as either pathologic evidence of Obliterative Bronchiolitis by biopsy or a decrease in Forced Expiratory Ventilation (FEV₁) greater than 20% compared to post transplant baseline values (22). FEV₁ is considered a reliable and consistent indicator of lung function. The consensus report for the staging of BOS in post lung transplant patients is described in I.A.2.1 Table 1, page 8

II.A.2 BRONCHOALVEOLAR LAVAGE (BALF)

Two ml aliquots of the BALF, taken from the right middle lobe for bilateral and right single lung transplants and from the lingula for left single transplants, was collected during surveillance bronchoscopies. Samples were snap frozen and stored at −80°C until analysis.

II.A.3 OxS MEASUREMENTS

II.A.3.1 BALF

II.A.3.1.1 Total Lipid Peroxides (LPO)
Free radicals (FR) are generated daily as part of normal aerobic metabolism (232). One of the free radicals generated is the \( \text{OH}^• \) radical. This is a toxic free radical, which attacks biological molecules including lipids by abstracting \( \text{H}_2 \) thus initiating lipid peroxidation (LPO) (233). End products of LPO such as malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) can be measured in plasma and BALF to determine OxS.

BALF was analyzed for total lipid peroxides using a commercially available kit (LPO 586, Oxis International™, Portland, USA), which measures free malondialdehyde (MDA) and 4-hydroxyalkenals. This method is based on a reaction of N-methyl-2-phenylindole (R1) with MDA and 4-hydroxyalkenals at 45°C and sample absorbance are measured at 586 nm. Therefore, we measured two end products of LPO: malondialdehyde and 4- hydroxy-nonenol (4-HNE). It is of interest to measure both procedures, as 4-HNE may be more toxic in vivo, than MDA (234). This is also a well recognized and validated method used previously in our lab (227, 235-239).

Another marker of LPO arising from the peroxidation of arachidonic acid (240) \( \text{F}_2 \)-isoprostanes, was not measured in our study. Although it is a recognized method (241,242), it is more tedious and requires more time to measure.

The following is the procedure to measure MDA and HAE.
**Assay Procedure for MDA + HAE (MSA Solvent Procedure):**

1. Standards were prepared according to Table 1 (see below) in clean glass test tubes. Standards were run in duplicate.

2. 200 µL of sample was added to the clean glass test tube.

3. 650 µL of diluent R1 reagent was added to sample.

4. Sample was vortexed gently.

5. 150 µL R2 reagent was added.

6. The samples and standards were mixed well and covered.

7. Then the samples were incubated at 45°C for 60 minutes.

8. Centrifuge turbid samples (15,000 x g for 10 minutes) to obtain a clear supernatant. (Allegra, Mississauga, Canada)

9. The clear supernatant was transferred to a cuvette.

10. Absorbance was measured at 586 nm* on DU-spectrophotometer

*The color is stable for at least an hour at room temperature or 2 hr at 4°C if the samples are stored in the dark and no evaporation occurs.

The coefficient of variation for within runs for MDA is 4.1%.

**Sample Blank (Abs)**

A sample blank was measured to correct for any $A_{586}$ contribution due to the sample. This blank was made by adding 650 µL of 75% acetonitrile/25% Diluent instead of the diluent R1 reagent to the assay tube. The acid addition and sample incubation steps were then carried out as described above.
Calculations

1. Using the standard data, calculate the net $A_{586}$ for each standard by subtracting the blank ($A_0$) value from each of the standard $A_{586}$ values. Plot net $A_{586}$ vs [MDA], and perform a linear regression analysis of $A_{586}$ on [MDA]:

$$[\text{MDA}] = a[\text{MDA}] + b$$

2. Calculate the concentration of analyte in each unknown from the net $A_{586}$ of the sample (if a sample blank was required, subtract $A_{50}$ from the net sample absorbance.):

$$[\text{MDA}] = \frac{A_{586} - b \cdot df}{a}$$

Where:

- [MDA] is the $\mu$M concentration of MDA in the sample
- $A_{586} = $ Net absorbance at 586 nm of the sample

- $a=$ regression coefficient (slope)
- $b=$ intercept
- $df=$ dilution factor

II.A.3.1.1 Figure 1: Example of a standard Curve

![MDA Standard curve](image-url)
There is no single biomarker that is considered the ‘gold standard’ measurement of lipid peroxidation (LPO). A solution to this problem is to use 2 techniques of measurements (243). Therefore, in addition to LPO we also measured BALF oxidized glutathione.

**II.A.3.1.2 Oxidized glutathione (GSSG)**

We measured Oxidized glutathione (GSSG) in BALF by using the NWLSS™ Glutathione Assay kit (NorthWest Life Science Specialties Inc, Vancouver, WA). Glutathione (GSH) exists in all cells, and is oxidized to GSSG in response to increased production of free radicals (244). The test principle of this kit is that DTNB reacts with GSH to form a chromophore TNB and GS-TNB. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH) releasing a second TMB molecule and recycling the GSH; thus amplifying the response. Any GSSG initially present in the reaction mixture or formed from the mixed disulfide
reaction of GSH with GS-TNF is rapidly reduced to GSH. To remove all the GSH and obtain GSSG, 4-vinylpyridine was added to the samples. Only BALF samples were analyzed for GSSG.

This method has been used frequently in other studies (57, 160, 245-247) and it is considered a good index of lipid peroxidation (248).

**PROCEDURE FOR QUANTITATIVE MEASUREMENT OF GSSG**

1. BALF samples were thawed for 2 hours in refrigerator
2. Microplate was removed from package
3. The GSH Calibrators were diluted 1:19 (1/20) in Assay Buffer.
4. 250 µL sample or diluted Calibrator was combined with 250 part cold 5% meta-phosphoric acid
5. Samples were centrifuged to remove precipitated protein at 1000 x g; 5 minutes (Centrifuge: Allegra, Mississauga, Canada)
6. 200 µL supernatant was collected and placed in a second tube
7. 10 µL 4 N NaOH was added and mixed
8. Tubes were placed on ice
9. 200 µL neutralized sample and Calibrator was transferred to tubes labeled ‘GSSG’
10. 10 µL 1 M 4-vinylpyridine was added to remove the reduced GSH
11. GSSG tubes were held for 60 minutes at room temperature
Using the NWK-GSH01™ High Sensitivity Protocol to measure GSSG the following procedure was followed:

- 50 µL calibrator or sample was added to microplate well
- 50 µL DTNB was then added
- followed by adding 50 µL GR
- then 50 µL NADPH was added

Microplate reader was set to read at Wavelength-405 nm (Reader:Ch-4051 Basel Switzerland)
- Mode=Kinetic
- Reaction Time=3 minutes
- Read Interval=30-60 seconds for 10 minutes

**Abbreviations**
- NAOH: sodium hydroxide
- DTNB: 5-5-Dithiobis (2-nitrobenzoic acid) in phosphate buffer with EDTA
- GR: Glutathione Reductase
- NADPH: β-Nicotinamide adenine dinucleotide phosphate, reduced

A calibration curve was generated plotting the increase in the rate ($\Delta A_{405}/\text{minute}$) as a function of the concentration of GSSG.

In addition, we also assessed antioxidants, to determine if the antioxidant defense system was affected by the degree of lipid peroxidation.
II.A.3.1.3 Antioxidant potential (AOP)

BALF AOP was assessed using a commercially available kit, Bioxytech AOP-490™. This assay is based upon the reduction of Cu⁰²⁺ to Cu⁰ by the combined action of all antioxidants such as bilirubin, albumin, vitamin E, ascorbic Acid, uric acid and glutathione present in the sample. A chromogenic reagent, Bathocuporine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu⁰ which has a maximum absorbance at 490nm. A standard curve was created using known concentrations of uric acid (a water soluble antioxidant). Results are expressed as “mM uric acid equivalents.”

Measuring the total AOP of the system may have more benefits than measuring individual antioxidants (249,250) as this method can assess globally the ability of biological fluids (BALF and plasma) to handle OxS (251). The downside is that by measuring a global perspective of antioxidants, individual antioxidant levels are not known, and as such, if one or more are increased or decreased then the overall result will indicate no change. Due to limited amount of BALF we were unable to measure individual BALF antioxidant levels in our patient population.

The detailed procedure to determine AOP is as follows:
The uric acid standard was reconstituted with 1.5 ml deionized water, which was added directly to the vial. This solution was then vortexed for 30-60 seconds to dissolve the standard. The uric acid concentration of this solution is 2.0 mM. Serial dilutions of the 2.0 mM uric acid standards to obtain six standard levels to calibrate a calibration curve were conducted. (Oxis Research, Portland OR.)

2.0 mM: No change to solution
1.0 mM: Add 500 µl of 2.0 mM standard to 500 µl deionized water
0.5 mM: Add 500 µl of 1.0 mM standard to 500 µl deionized water
0.25 mM: Add 500 µl of 0.5 mM standard to 500 µl deionized water
0.125 mM: Add 500 µl of 0.25 mM standard to 500 µl deionized water
0.0 mM: Only 500 µl deionized water

Procedure:

1. Samples were thawed in refrigerator for 1 hour
2. Test tube for each replicate of the standards and samples were labeled (in duplicate)
3. Each standard or sample was diluted 1/40 with RI (15 µl plus 585 µl R1 was used)
4. 200 µl of each was added into microplate well
5. Plate was read at 490 nm for a reference measurement
6. Results were printed for initial absorbance reading
7. 50 µl of R2 was then added to each well and mixed (use the ‘mix’ option on microplate reader (Reader: CH-4051, Basel Switzerland)
8. Incubated for 3 minutes at room temperature (use stop watch)

9. 50 µl Stop Solution was added and mixed well (again, the 'mix’
    option on microplate reader was set up)

10. Plate was read again at 490 nm (Ch-4051, Basel Switzerland)

11. Results for final absorbance reading were printed

Calculations

The net absorption was calculated by subtracting the initial
absorbance obtained in Step 5, from the last absorption reading (Step
10). The net absorption at 490 was then plotted for each level of
standard versus the uric acid concentration. A standard curve was
then generated (see example).
II.A.3.1.4 BALF correction

Biomarkers collected in BALF are diluted by the procedure, which involves the use of a certain volume of normal saline for the lavage. Although not completely accepted in the literature, a dilution factor has been suggested to correct for the volume of normal saline used to collect BALF specimens. One of the methods supported is the measurement of urea.

II.A.3.1.4.1 Dilution Correction Methodology

This method normalizes for variations in the volume of BALF returns between patients, and has been validated to correct for the effects of dilution by comparing the concentration of urea present in the BALF with that in the serum (252). This dilution factor is then
used to correct for all the variables measured in BALF (252). Urea is believed to be a reliable measure as it diffuses readily through the body; does not have a net charge at physiologic pH; is not consumed or produced by lung cells and plasma and epithelial lung fluid urea concentrations are similar. The disadvantage of using urea is that urea from other sources (blood and tissue) may diffuse into the recovered lavage fluid during the procedure. However, this diffusion can be minimized by decreasing ‘dwell times’ (defined as the amount of time the fluid instilled remains in the lung prior to being lavaged) and by using standardized lavage procedures (253), which is the current protocol followed at UHN. However, using urea as an internal marker to assess dilution is not widely accepted by all authors (254).

Thus, although using urea may not reflect the true correction factor (not a gold standard), it may be the best of imperfect methods to use (255). Therefore, until a gold standard is defined, we reported both the diluted and undiluted results (253).

II.A.3.1.4.2 Urea methodology

Urea content of BALF was measured by colorimetric assay using a commercially available kit (1:1 sample (v/v): reagent BUN Infinity™, Therma Diagnostics, Louisville, CO, U.S.A).

The procedure for determining urea in BALF is as follows:

1. Water bath was set at 37°C
2. Samples were removed from freezer and placed in fridge for 2 hours
3. The DU-640 Beckman spectrophotometer wavelength was set at 340 nm (Corona, California)
4. The appropriate cuvettes for the spectrophotometer (square with 1 ml volume) were used
5. The standards were measured (see below)
6. The blank was measured.
7. Reagent blank was used to zero the readings
8. Reagent blank was prepared using 300 µL BUN reagent™ and 3 µL deionized water.
9. For each sample, 300 µL BUN reagent was dispensed into cuvettes and warmed to reaction temperature (37ºC) for approximately 5 minutes.
10. 3 µL of sample or standard was added to its respective tube and mixed gently (1:1) (v:v).
11. Samples/standards were incubated for 30 seconds (stop watch); at reaction temperature, the initial absorbance was measured and recorded.
12. After an additional 60 seconds (stop watch) a second absorbance was read and recorded.
The amount of urea in the sample was calculated using the following equation:

\[ 40 \times \frac{\Delta \text{abs/minute of sample}}{\Delta \text{abs/minute of calibrator}}/20 \text{ (mg/dl)} \]

(Infinity™ Urea Liquid Stable Reagent, division of Thermo Electron Corporation).

This test was performed as a kinetic assay in which the initial rate of the reaction is linear for a limited amount of time. Urea in the sample is hydrolyzed by urease to ammonia and carbon dioxide. The second reaction, catalyzed by glutamate dehydrogenase (GLD) converts ammonia and α-ketoglutarate to glutamate and water with the concurrent oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). Two moles of NADH are oxidized for each mole of urea present. The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample.

**II. A.3.1.4.3 Serum urea**

Serum Urea was analyzed at the Toronto Medical Laboratory, University Health Network (UHN).

**II.A.3.2 Plasma**

For plasma measures, blood was collected in EDTA-containing tubes and promptly centrifuged at 910 x g; 10 minutes. Plasma was then removed and frozen at -80°C until analysis. This same
methodology for plasma analysis was conducted for both lung and liver transplant recipients.

**II.A.3.2.1 Antioxidant Potential (AOP)**

AOP was also assessed in the plasma using a commercially available kit, Bioxytech AOP-490™ (Oxis Research, Portland, USA), as per methodology described in section II.A.3.2.1.page77.

**II.A.4 Antioxidant Micronutrients**

**II.A.4.1 Vitamin C**

Vitamin C is an important hydrophilic antioxidant and acts as a reducing agent (256) and can also directly scavenge $\text{O}_2^-$ and $\text{OH}^\bullet$ by forming the semidehydroascorbate free radical that is subsequently reduced by glutathione (GSH) (256). In humans both the reduced form of Vitamin C (L-ascorbic acid) and the oxidized form (L-dehydroascorbic acid) are biologically active and can be measured (257).

For these assays, 0.5 ml of plasma was stabilized immediately with 0.5 ml 100g HPO$_3$ (meta-phosphoric acid)/L prior to freezing and this technique has shown that samples are stable at $-70^\circ$ for several years (258). The thawed serum samples were analyzed by spectrophotometry using 2, 4,-dinitro-phenylhydrazine as the chromogen. This method has been modified to comply with the current procedure used by Clinical Chemistry Division, Centers for
Disease Control for the Second Health and Nutrition Examination Survey (NHANES II) 1976-1980 (259,260) and is a validated method for measuring total Vitamin C.

Ascorbic acid is converted to dehydroascorbic acid in the presence of thiourea and copper sulfate. Dehydroascorbic acid couples with 2, 4-dinitrophenyl-hydrazine in 9.0 mol/L sulfuric acid to form a bis-2, 4-dinitrophenylhydrazine derivative. When treated with 65% (v/v) sulfuric acid, this derivative yields a stable brownish-red color, which is measured with a spectrophotometer at 520 nm.

We compared our plasma vitamin C levels with levels obtained from the (260) NHANES I study and other studies (261). Our results displayed similar levels and roughly the same standard deviations (SD). Wide variations in SD are normal and expected based on changes in the consumption of vitamin C containing foods, along with seasonal variations (262). Furthermore, correlations between dietary vitamin C intake and plasma levels have been noted in some patient populations (263).

Detailed Vitamin C assay procedure is as follows:
1. Incubator was set 20°C and allowed to equilibrate.
2. Frozen extracts were removed from freezer and allowed to thaw at room temperature and were used immediately.
3. Specimens and controls were centrifuged at 910 x g; 10 mins, to remove proteins and obtain the clear supernatant for analysis.
4. In duplicate, 400 µl of sample extract or control specimen were pipetted into a clean test tube.
5. 135 µl of color reagent was added to each sample tube and mixed by vortex.
6. Standards were prepared. For analysis, standards were prepared in triplicate.
7. After mixing, tubes were covered with clear wrap and rubber band and incubated for 20 hr at 28°C, in water bath Cornelius, U.S.A.
8. After incubation, tubes were moved and placed in an ice bath for 15 minutes and then 650 µl of 65% sulfuric acid was added to each tube, mixed well by vortex.
9. Tubes stood at room temperature for 30 minutes before measuring their absorbance levels. The yellow-orange color that develops during this 30-minute incubation is stable for at least 2 hr. 1-cm square cuvettes were used.
10. Absorbance was determined at 521 nm using 5% MPA (65% sulfuric acid as the analysis blank, representing Standard 0). The coefficient of variation for within runs for Vitamin C is 3.79%.
A detailed overview of reagents and standards preparation is contained in Appendix 1.
II.A.4.2 Vitamin E and Carotenoids

Alpha-and gamma-tocopherol, beta-carotene and retinol were analyzed by High performance liquid chromatography (HPLC) using a reverse phase column and fluorescence spectrophotometry according to the validated method of Stacewicz-Sapuntzakis (264). This method has been used in our laboratory in patients with a variety of disease conditions (227, 235-239, 265). The within day and between day reproducibility of this method were 96.0 and 94.2% respectively. Similar plasma α-tocopherol levels have been reported in renal transplant patients (266) and our results appear to be within the same levels in other patient populations (267). Lipid and fat-soluble concentrations in plasma are positively correlated. Therefore an adjustment of fat-soluble antioxidant concentrations to lipid content in plasma was conducted to decrease any confounding effect that the lipids may exert on the vitamin concentrations (268).

The detailed procedure to measure tocopherols and carotenoids is as follows:
1. 400 µl of HPLC-grade ethanol containing beta-hydroxy-toluene (BHT) (35 mg/100ml) as an antioxidant were added to 200 µl of plasma. This was to precipitate the proteins in the sample.
2. One ml HPLC-grade n-hexane was added and the sample was mixed vigorously for 10 min with a vortex.
3. The sample was centrifuged (910 x g; 10 min), resulting in a separation of the water phase (bottom) and the organic phase (top layer). The top layer was transferred into a pre-weighed test tube with a pasteur pipette.

4. To increase the lipid extraction, another 1 ml of hexane was added to the test tube containing the water phase, followed by brief vortex and centrifugation (910 x g; 10 min). The top layer was transferred and added to the previously separated lipid phase.

5. The hexane was evaporated under a gentle stream of nitrogen gas.

6. The test tube containing the residue (lipid) was weighed to calculate the amount of lipid in the tube.

7. The lipid was re-constituted in 50 µl of stabilized ether and 150 µl of 95% HPLC-grade methanol.

8. The sample was injected into the HPLC system using an autosampler. The mobile phase was 95% HPLC-grade methanol for the first 2.5 minutes and pure 100% HPLC-grade methanol for the remainder of the run.

9. Peaks were separated using a reverse-phased C18 column at flow rate of 1.0 ml/min.

10. A Varian programmable multi-wavelength detector was used to detect tocopherols, retinol and carotenoids at 292, 325, and 450 nm respectively (269).
This HPLC methodology has been determined as a precise, accurate, selective, sensitive and reproducible method (270). For information on additional chemicals and standards for tocopherols and carotenoids refer to Appendix 2.

**II.A.5 Nutrition profile**

OxS can be influenced by dietary intakes and nutritional status.

**II.A.5.1 Intake**

**II.A.5.1.1 Food Record Collection**

As part of the transplant protocol each patient regularly underwent dietary assessment during scheduled transplant clinic appointments.

For dietary intake, instructions were given on how to keep a food record for either three or seven days by providing forms that were designed to collect the following information: the particular food item, a description of the food item including the brand, type, preparation method and the number of servings consumed upon entry to the study. Subjects estimated the edible portions of consumed foods using the National Cholesterol Education two-dimensional food portion visual chart (271). This chart has been validated previously for use in adult men and women 20-70+ years of age (272).

The method of the seven-day dietary record was evaluated in other studies and found to be valid and reliable for use in clinical trials,
as well as for the assessment of micronutrient intakes (273,274). The advantages of the seven-day food record include that it does not rely on memory, it is easy to quantify the amounts, and can, over several days accurately represent usual intake (FAO/WHO, 1996). However the limitations of recording intake over seven-days include: high participation burden, subject literacy, and incorrect estimation of portion sizes (275). As well, subjects may alter their intake during the time of recording (FAO/WHO, 1996). To minimize some of the above-mentioned limitations, research subjects were called to review their daily intake. As well, a review and clarification of the seven-day record was completed during the assessment interview.

Food records were then analyzed using computer software (Diet Analysis Plus, version 8.0.1, Thomson Wadsworth 2006, Connecticut, USA). The acceptable macronutrient distribution range for adults older than 18 was considered to be: 20-35% fat, 45-65% carbohydrates and 10-35% protein. To estimate the prevalence of inadequate intakes, the Estimated Average Requirement (EAR) as a cut-point was used. The distribution of intakes were determined by examining the proportion above and below the EAR (276-282).

**II.A.5.2 Nutrition Assessment**

**II.A.5.2.1 Anthropometry**

The dietitian performed all anthropometrics.
II.A.5.2.1.1 Body Mass Index (BMI)

Body weight and height were measured using an upright scale (Continental Scale Corp, Chicago, Ill) and BMI was electronically calculated by dividing weight (kg) by height (m) squared. Only BMI was calculated for the lung transplant recipients.
II.B. OxS AND HCV LIVER RECIPIENTS

II.B.1 LIVER BIOPSY PROCEDURE

A biopsy needle is percutaneously inserted through the abdomen into the liver via an ultrasound-guided procedure to obtain a piece of liver tissue. During the procedure, patients are supine and asked to exhale and hold their breath as needle was inserted and a liver sample is withdrawn (283).

II.B.2 DEFINITION HCV DISEASE RECURRENTNESS

For the purpose of our study HCV disease recurrence was defined based on a liver biopsy indicating only mild fibrosis (injury) in the absence of other causes such as rejection, other viral infections or drug toxicity. The rationale for this, is that current UHN protocol indicates when biopsy results show fibrosis $\geq$ Stage 1 with any activity or activity defined as Grade 3 with fibrosis any Stage or fibrosing cholestatic recurrent HCV, antiviral treatment is initiated. Thus, this would maintain a homogenous patient population and prevent any possible confounding effect from antiviral medications.

II.B.3 LIVER TISSUE METHODOLOGY

HCV-LT had surveillance biopsies at approximately six-month post transplant. Necroinflammation grading and fibrosis stage were
scored according to METAVIR (284). Diagnostic biopsies for control patients and routine biopsies were conducted on HCV-NT patients. For the controls and HCV-NT patients, histological scoring was used to assess fibrosis, inflammation and degree of hepatic steatosis using the Brunt system (285).

**II.B.3.1 Liver Tissue Measurements**

Liver tissue was frozen in liquid nitrogen within 15 minutes of collection and stored at -80°C until analysis. Liver tissue was processed by thawing the samples; labeling and weighing glass test tubes (average of 3 weights was taken); liver tissue was placed in test tube weighed again 3 times and average was taken. Then the tissue was homogenized in 290 µl ice-cold 20 mM PBS buffer, pH 7.3 with 10 µl of 5mM butylated-hydroxy-toluene to avoid ex-vivo oxidation. Following this, the suspension was centrifuged and supernatant was fractioned for analysis of LPO (200 µl) and AOP (15 µl).

**II.B.3.1.1 Total Lipid Peroxides (LPO)**

Lipid hydroperoxides were assessed using a commercially available kit (LPO 586, Oxis International, Portland, USA), which measures free malondialdehyde (MDA) and 4-hydroxyalkenals. This method is based on a reaction of N-methyl-2-phenylindole with MDA and 4-hydroxyalkenals at 45 °C and read at absorbance of 586 nm
The detailed procedures for analyzing LPO has been described previously, please refer to section II.A.3.1.1 page 64.

**II.B.3.1.2 Antioxidant Potential (AOP)**

Antioxidant potential (AOP) was assessed using a commercially available kit (Oxis Research, Portland, USA). This assay is based upon the reduction of Cu$^{++}$ to Cu$^{+}$ by the combined action of all antioxidants present in the sample. A standard of known uric acid (a water soluble antioxidant) concentration was used to create a calibration curve. The results are expressed as “mM uric acid equivalents”. For the detailed procedure please refer to section II.A.3.1.3 page 70

**II.B.4 Plasma**

Measurements of AOP, Vitamin C, tocopherols, and carotenoids followed the same methodology as described in the previous section on OxS in BOS lung recipients. Please refer to section II.A.3.2 page 76.

**II.B.5 Nutrition Profile**

**II.B.5.1 Intake**

As part of the liver transplant protocol, each patient was assessed during regularly scheduled transplant clinic appointments. In this population, a three-day food record was used because HCV patients were less compliant with a seven-day food record.

For a full description of food record methodology please refer to section II.A.5.1.1, page 81.
The three-day food records were collected the same way as the seven-day food records and were analyzed using computer software (Diet Analysis Plus, version 8.0.1, Thomson Wadsworth 2006). For detailed discussion on food record analysis, please refer to section: II.A.5.1.1 page 82.

**II.B.5.2 ANTHROPOMETRY**

**II.B.5.2.1 BMI**

Anthropometric measurements on patients were performed. For BMI description please refer to section II.A.5.2.1. page 83.

**II.B.5.2.2 Waist-to-Hip Ratio (WHR)**

In addition waist and Waist-to-hip ratio (WHR) were measured. Waist Circumference was measured by placing a measuring tape horizontally around the waist at the narrowest part of the torso (for obese subjects at the umbilicus) (286). Hip circumference was measured by using a measuring tape placed horizontally at the greatest part of the buttocks.

For this, all subjects stood erect with the abdomen relaxed, arms at their side, feet together and weight equally divided over both legs. Locating and marking the margin of the lowest rib completed the waist measurement; this was palpated and marked at the iliac crest in the midaxillary line. An elastic tape was applied horizontally midway between the lowest rib margin and the iliac crest. The tape was tied
firmly so it stayed in position around the abdomen about the level of the umbilicus. The waist circumference was measured to the nearest mm by positioning the fiberglass tape over the elastic tape. Subjects were asked to breathe normally and to breathe out gently at time of the measurement, to prevent subjects from contracting muscles or from holding their breath. The hip circumference was measured at the point yielding the maximum circumference over the buttocks with the tape held in a horizontal plane, touching the skin but not indenting the soft tissue. Waist-to-hip ratio was then calculated (286).

II.B.5.2.3 Bioelectric impedance analysis (BIA)

Body fat was estimated using bioelectric impedance analysis (BIA) (287). BIA includes measurements of resistance and reactance by a 4-terminal impedance analyzer (model BIA-103, RJL-system, Detroit, Mich) using a single-frequency 800 µA current at 50 kHz while the subjects were supine with arms and legs extended (288). Fat free mass was estimated using the prediction equation contained in the manufacturer’s software, which incorporates information on age, sex, height, and weight. Body fat was then calculated by subtracting the fat-free mass from the total weight (289-291).

For this, participants wore light clothing and were barefoot (or removed the shoe and sock from the right foot). The subject reclined in a supine position on the measuring table with arms adjacent to, but
not touching the body, palms flat against the table, and legs adjacent to each other but not touching. Four surface self-adhesive spot electrodes were placed on the dorsal surface (top) of the right hand and the dorsal surface of the right foot. Prior to placement of the electrodes the skin was wiped with alcohol at the 4 locations for electrode placement. Resistance and reactance were determined on the right side of the body.
CHAPTER III. Oxs AND BOS LUNG RECIPIENTS

III.1 ARTICLE: Oxs AND NUTRITIONAL INTAKE IN LUNG PATIENTS WITH BRONCHIOLITIS OBLITERANS SYNDROME (BOS).

by

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This work was presented in a poster format at

- Allied Health Research Day, Toronto, Ontario, Canada, Jan 31, 2008 (partial funding provided by Allied Health, UHN)
- Ontario Respiratory Care Society, Toronto, Ontario, Canada, Feb 3, 2008 (partial funding provided by ORCS, Toronto, ON)

This work was presented in a podium format at

- Day in Transplant, UHN Conference, Toronto, Ontario, Canada, Dec 8, 2007
- CTS/CAT International Conference, Mt. Tremblant, Quebec
  - won the Wyeth Scientific Award for best podium presentation.
III.1.1 ABSTRACT

Survival after lung transplantation is limited by Bronchiolitis Obliterans Syndrome (BOS). OxS (OxS) can be associated with BOS due to chronic inflammation. The type of fat and antioxidant intakes may also contribute to OxS. The aim was to measure and compare OxS and nutritional intakes in non-BOS and various stages of BOS patients. Fifty-eight lung recipients with and without BOS were prospectively enrolled and classified as: non-BOS; BOS Op-1 (mild) and BOS 2-3 (severe). Nutritional intake and plasma Vitamin A, C and E were measured. In a subgroup of 37 patients OxS was assessed by measuring lipid peroxidation (LPO µM MDA) and oxidized glutathione (GSSG) in bronchoalveolar lavage BAL fluid (BALF). One-way ANOVA was used to compare groups. Results are reported as Mean ± SEM. There was no significant difference in demography and time post transplant among groups. Although cell counts were comparable in BALF, severe BOS patients had significantly higher BALF LPO concentrations when compared to milder stage of BOS or non-BOS (p=0.001, for both). Severe BOS recipients also had higher BALF GSSG concentrations compared to milder stage of BOS (p=0.001) or non-BOS (p=0.007).

Conclusion: Patients with severe BOS are more oxidatively stressed compared to mild and non-BOS recipients.
III.1.2 Introduction

Lung transplantation has an excellent one-year survival rate of 80%. However, at 5-years the survival rate decreases to 50% due to chronic rejection, manifested as Bronchiolitis Obliterans Syndrome (BOS) (292). BOS contributes significantly to increased morbidity and mortality, and is the primary limiting factor in long-term survival post lung transplantation. BOS is marked by decreases in FEV$_1$ from baseline and is graded according to criteria set out by International Society Heart and Lung Transplantation (ISHLT) (22).

The pathogenesis of BOS involves both alloimmune-dependent and -independent mechanisms (23). The most highly associated risk factor for the development of BOS is acute rejection in terms of both the number and the severity of rejection episodes (48,293, 294). Other risk factors that may be associated with the development of BOS include: lymphocytic bronchitis/bronchiolitis (LB/B) (295) Cytomegalovirus (CMV) infections (38), and gastroesophageal reflux disease (GERD) (296). All these factors associated with BOS are characterized by chronic or acute inflammation in the transplanted lung (59). In BOS patients, Bronchoalveolar Lavage Fluid (BALF) also shows increased levels of pro-inflammatory cytokines like Interleukin-8 (IL-8) in addition to increased myeloperoxidase (MPO) (a pro-oxidative enzyme) (297) and neutrophils (60). In addition to MPO, these
activated neutrophils also produce reactive oxygen species (ROS) that can lead to further tissue damage and contribute to the development of BOS.

Excess ROS may also produce an imbalance between prooxidants and the antioxidant defense system, leading to Oxidative Stress (OxS). OxS can be assessed by various laboratory analyses, for example by measuring by-products of lipid, protein and DNA oxidation. These include, lipid peroxidation metabolites such as plasma/tissue malondialdehyde (MDA) and 8-isoprostanes (13); protein oxidation parameters such as protein carbonyls (298) total thiols, advanced oxidation protein products and nitrotyrosine (14,15) and DNA damage {DNA strand breaks and guanine oxidation products (8-OHdG) (299). The antioxidant system can also be assessed by measuring antioxidant enzymes like glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) or by analysis of micronutrients such as antioxidant Vitamin E, C or carotenoids.

OxS may also be influenced by nutritional factors. High intake of certain polyunsaturated fatty acids (PUFA) may increase OxS because PUFAs are more susceptible to lipid peroxidation due to their multiple double-bonds (300) and high PUFA intake may increase the requirements for antioxidants (84). In addition, low intakes of antioxidants such as vitamin E, C carotenoids or selenium can also
weaken the antioxidant defense mechanism especially if there is a chronic state of inflammation, contributing to OxS (30-304). Therefore, considering that OxS can be associated with inflammation and tissue damage in BOS and that it can be influenced by dietary factors, the aim of this study was to measure and compare OxS and nutritional intake in lung transplant patients with and without BOS.

III.1.3 Material and Methods

III.1.3.1 Study design

This was a cross-sectional investigation of lung transplant patients consecutively recruited from April 2005 to October 2006.

III.1.3.2 Study Participants

Lung transplant recipients attending the outpatient lung transplant clinic at UHN were approached to participate in this study. Sixty-six patients were eligible for the study. Three patients declined to participate, three did not attend their scheduled clinic appointment and two patients were too sick to participate. Fifty-eight patients were recruited from the ambulatory lung transplant clinic at the Toronto General Hospital, 37 of whom were between 12-24 months (plasma and BALF available) and 21 of whom were >24 months (plasma values only) post transplant. Patients were eligible to participate if they were >18 years of age, attended the clinic, and were clinically and medically
stable. Participants were excluded if they had an infection at the time of recruitment.

**III.1.3.3 Measurements**

**III.1.3.3.1 BMI**

Patients’ height and weight were measured and BMI was calculated by dividing weight (kg) by height in meters squared.

**III.1.3.3.2 Food Record**

All patients were instructed to complete a seven-day food record using standardized portion size guides (305). As well, 24-hr dietary recalls and food frequencies were completed providing other validated instruments to determine nutritional intakes (277).

**III.1.3.4 Sample Collection**

**III.1.3.4.1 Bronchoalveolar lavage (BALF)**

BALF was performed as part of the routine surveillance bronchoscopies in patients who had their lung transplant within 24 months. This procedure is normally performed at 3, 6, 9, 12, 18 and 24 months post transplant. For this study, a sample from one BALF was consecutively collected in a subset of 37 patients. For the BALF procedure, two of 50 ml each, aliquots of normal saline were instilled into the right middle lobe of double lung and single right lung transplant recipients, with typical returns of 15-35 ml per 50 ml aliquot. Two ml of the BALF were snap frozen in liquid nitrogen (–
90°C) and stored at –80 °C until analysis. Serum and BALF samples were collected within 24 hours of each other.

**III.1.3.4.2 Serum**

Serum samples were collected from lung recipients in a fasting state. Blood was collected within 24 hours of bronchoscopy, and collected in EDTA-containing tube and promptly centrifuged at 910 x g; 10 minutes. Plasma was removed and frozen at -80°C until analysis.

**III.1.3.5 BALF Dilution Correction**

To normalize for variations in the volume of BALF returns between patients, we used a validated method (252) to correct for the effects of dilution by comparing the concentration of urea present in the BALF with that in the serum. Urea content of BALF was measured by colorimetric assay using a commercially available kit (1:1 sample: reagent BUN Infinity, Therma Diagnostics). Briefly, 300 µl of BALF sample and 300 µl reagent were combined for one minute. Consumption of NADH, a reflection of urea concentration, was then measured as change in absorbance at 340nm using a DU-640 Beckman spectrophotometer. Serum urea was measured using standard techniques. The correction factor for dilution of BALF was calculated by serum urea by BALF urea (252). This dilution factor was then used to correct all the variables measured in BALF in this study.
***III.1.3.6 OxS Measurements***

**III.1.3.6.1 BALF**

**III.1.3.6.1.1 Total Lipid Peroxides (LPO)**

BALF was analyzed for total lipid peroxides using a commercially available kit (LPO 586, Oxis International, Portland, USA), which measures free malondialdehyde (MDA) and 4-hydroxyalkenals. This method is based on a reaction of N-methyl-2-phenylindole (R1) with MDA and 4-hydroxyalkenals at 45°C and sample absorbance are measured at 586 nm.

**III.1.3.6.1.2 Oxidized glutathione (GSSG)**

Oxidized glutathione (GSSG) was measured by the NWLSS Glutathione Assay kit (NorthWest Life Science Specialties Inc, Vancouver, WA). Only BALF samples were analyzed. Briefly 250 µL of BALF sample was mixed with 250 µL cold 5% meta-phosphoric acid (MPA). Following centrifugation at 1000 x g for 5 minutes, 200 µL of supernatant was then mixed with 20 µL 4 N NaOH. 200µL of the neutralized MPA extract was then mixed with 10 µL 1M 4-VP (vinylpyridine) to eliminate all the reduced glutathione, leaving only the oxidized glutathione. These samples were held at room temperature for 1 hour. Following this, 50 µL of BALF sample was mixed with 50 µL DTNB; 50 µL Glutathione Reductase (GR) and 50 µL NADPH. Sample
absorbance was read spectrophotometrically at 405 nm for 10 minutes at 30-60 second intervals.

**III.1.3.7 Antioxidant Measurements**

**III.1.3.7.1 BALF**

**III.1.3.7.1.1 Antioxidant potential (AOP)**

BALF AOP was assessed using a commercially available kit, Bioxytech AOP-490. This assay is based upon the reduction of Cu$^{++}$ to Cu$^{+}$ by the combined action of all antioxidants such as bilirubin, albumin, vitamin E, ascorbic acid, uric acid and glutathione present in the sample. A chromogenic reagent, Bathocuporine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, selectively forms a 2:1 complex with Cu$^{+}$ which has a maximum absorbance at 490nm. A standard curve is created using known concentrations of uric acid (a water soluble antioxidant). For this reason, the results are expressed as “μmols uric acid equivalents” (UAE).

**III.1.3.7.2 Plasma**

**III.1.3.7.2.1 AOP**

Plasma AOP was assessed using commercially available kit Bioxytech AOP-490, described above.

**III.1.3.7.2.2 Vitamin C**

For these assays, 0.5 ml of plasma was stabilized immediately with 0.5 ml 100g (1:1) (v:v) HPO$_3$ (meta-phosphoric acid)/L prior to
freezing. The thawed serum samples were analyzed by spectrophotometry using 2, 4,-dinitro-phenylhydrazine as the chromogen (306).

**III.1.3.7.2.3 Tocopherols and Carotenoids**

Alpha- and gamma-tocopherol and retinol were analyzed by HPLC and fluorescence spectrophotometry. The method involves a reverse-phased C18-column to be used with an isocratic solvent system (methanol:acetonitrile:tetrahydrofuran, 50:45:5, by volume) after hexane extraction with 200 µl of plasma. Using a Waters 490 Programmable Multiwavelength Detector, retinol was detected at 325 nm, and α- and gamma-tocopherol were detected at 295 nm (269). β-carotene was also analyzed by HPLC and fluorescence spectrophotometer in a subset of 16 recipients. Sixty percent of the patients had undetectable levels, and no further analysis was conducted.

**III.1.4 Analysis of food record**

The data were analyzed for nutrient intake using the analysis program Diet Analysis Plus Version 8.0; 2006. To estimate the prevalence of adequate intakes, the Estimated Average Requirement (EAR) as a cut-point was used. The distributions of intakes were determined by examining the proportion above and below the EAR (307).
III.1.5 Ethics Approval

This study was performed according to the guidelines of the 1975 Declaration of Helsinki and was approved by the Research Ethics Board, University of Health Network, Toronto, Ontario, Canada (Appendix 3).

III.1.6 Statistical Analysis

Data are expressed as Mean ± SEM. For each variable, a comparison of means was completed. If the variable displayed normal distribution, one-way ANOVA was utilized and Tukey’s post hoc test was conducted. Chi-square was conducted on categorical data. If data were not normally distributed, the equivalent Kruskal-Wallis test was used. SPSS 16.0 2007, was used for analysis, and statistical significance was defined as P<0.05.

III.1.7 Results

The characteristics of the 58 lung transplant recipients who were enrolled in the study are described in III.1.7.Table 1. The 37 patients with BALF measurements are described in III.1.7.Table 1a. Patients were classified in three groups, according to the changes from their baseline FEV₁: non-BOS; BOS Op-1; and BOS 2-3. There were no statistically significant differences in the three patient groups with respect to BMI, gender distribution, age, diabetes mellitus, and primary underlying lung disease, or type of lung transplant (single
versus bilateral), when analyzed with either the 58 patients or the 37 patients with BALF. In addition, there were no differences in time post transplant or cell count in BALF III.1.7.Table 1a.

### III.1.7.1 OxS Biomarkers

#### III.1.7.1.1 BALF LPO (n=37)

Patients with more severe BOS (BOS 2-3) had significantly higher LPO concentrations when compared to patients with milder stage of BOS (BOS Op-1) (p=0.001) or with non-BOS (p=0.001). There was no statistically significant difference in the BALF LPO between non-BOS and the milder stage of BOS (BOS Op-1 p=0.807). When we analyzed the raw data, without correcting for the dilution factor, no statistical differences were observed (III.1.7.Table 2).

#### III.1.7.1.2 BALF GSSG (n=37)

Patients with more severe BOS (BOS 2-3) had significantly higher corrected BALF GSSG concentrations when compared to patients with milder stage of BOS (BOS Op-1) (p=0.001) or with non-BOS (p=0.007). There was no statistically significant difference in the BALF GSSG between non-BOS and the milder stage of BOS (BOS Op-1 p=0.118). Again, when the raw data were compared without correcting for the dilution factor no statistical significance was observed.

### III.1.7.2 Antioxidants
**III.1.7.2.1 BALF AOP**

The corrected and uncorrected BALF AOP levels among the three groups were not statistically significantly different (p=.123; p=.162 respectively).

**III.1.7.2.2 Plasma AOP**

There was a trend toward a statistically significant difference in plasma levels of AOP among the three groups (p=.087). If the sample size had been greater, it would have been sufficiently powered to detect a significant difference.

Thus, assessment of OxS in BALF using two different biomarkers (LPO, GSSG) demonstrates that using the correction factor, patients with a more severe form of BOS have higher OxS in their lungs but that the antioxidant system was not significantly impaired when assessed by AOP.

**III.1.7.2.2 Plasma Antioxidant vitamins**

Overall, there were no statistically significant differences among the three patient groups with respect to the plasma levels of γ-tocopherol, α-tocopherol, retinol or ascorbic acid (Table 3). In all 3 groups plasma α-tocopherol:cholesterol was within the reference ranges(268). There was no significant difference among the three groups in terms of proportion of patients below the reference range (**III.1.7.2.2.Table 3**). In a subset of 16 patients β-
carotene was undetectable. Vitamin C was adequate in most of the patients.

**III.1.7.3 Nutritional Intakes and Status**

Analysis of the food records revealed no differences with respect to the intake of energy, protein, carbohydrates, total fat, PUFAs or fiber among the three groups (**III.1.7.2.2.Table 4**). Overall, these intakes are similar to the current Dietary Reference Intakes (DRI) and appear similar to Canadian population intakes (308,309). There were no significant differences in nutritional intakes of the micronutrient antioxidants vitamins A, C and E (Table 5) based on the Estimated Average Requirements (EAR). However while there was adequate intake of vitamin A and C in the majority of the patients, there was evidence of inadequate intake of vitamin E in a large proportion of the subjects in each group. Sixty three percent or more of the patients consumed adequate intakes of Vitamin A and C. However, 86%, 87% and 78% of the patients in non-BOS, BOS Op-1, BOS 2-3, respectively failed to meet their DRI for Vitamin E (**III.1.7.2.2.Table 5**). With the exception of CF patients, the majority of the patients were not taking vitamin and mineral supplements.
### III.1.7. Table 1. Demographics and Clinical Characteristics of 58 Lung Transplant Recipients

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS Mean ± SEM (n=23)</th>
<th>BOS Op-1 Mean ±SEM (n=18)</th>
<th>BOS 2-3 Mean ± SEM (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AGE (YEARS)</strong></td>
<td>49.0 ± 2.28</td>
<td>53.1 ± 2.66</td>
<td>51.3 ± 2.67</td>
</tr>
<tr>
<td><strong>GENDER (F/M)</strong></td>
<td>11f/12m</td>
<td>9f/9m</td>
<td>6f/11m</td>
</tr>
<tr>
<td><strong>DIABETES MELLITUS (N/%)</strong></td>
<td>11/48</td>
<td>6/33</td>
<td>10/59</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.6 ± 1.10</td>
<td>25.8 ± 1.12</td>
<td>26.4 ± 1.10</td>
</tr>
<tr>
<td>% PT BMI &gt;27(kg/m²)</td>
<td>50</td>
<td>41</td>
<td>44</td>
</tr>
<tr>
<td><strong>INDICATIONS FOR Tx</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>CF</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PF</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>PPH</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>OTHER</td>
<td>7</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><strong>TYPE OF TRANSPLANT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SINGLE LUNG</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>BILATERAL LUNG</td>
<td>19</td>
<td>12</td>
<td>11</td>
</tr>
</tbody>
</table>

**Abbreviations:** BMI: body mass index; COPD: chronic obstructive pulmonary disease; CF: cystic fibrosis; PF: pulmonary fibrosis; PPH: primary pulmonary hypertension; OTHER: Lymphangioleiomyomatosis, Eisenmengers, cryptogenic organizing pneumonia. No significance among the three groups using one-Way Anova with p<0.05 for statistical significance.
### III.1.7. Table 1a. Characteristics of 37 Lung Recipients Undergoing BALF Analysis

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS Mean ± SEM (n=15)</th>
<th>BOS Op-1 Mean ±SEM (n=12)</th>
<th>BOS 2-3 Mean ± SEM (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>50.7 ± 2.3</td>
<td>52.4 ± 3.8</td>
<td>55.4 ± 2.8</td>
</tr>
<tr>
<td><strong>Diabetes Mellitus (%)</strong></td>
<td>50</td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.3 ± 1.1</td>
<td>27.3 ± 1.3</td>
<td>27.2 ± 1.3</td>
</tr>
<tr>
<td><strong>Time from Tx: (months)</strong></td>
<td>21.8 ± 3.8</td>
<td>30.6 ± 5.6</td>
<td>36.0 ± 6.3</td>
</tr>
<tr>
<td><strong>BALF Differential Cell Counts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Macrophages</td>
<td>87.1 ± 5.1</td>
<td>86.2 ± 2.7</td>
<td>91.4 ± 2.3</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>3.6 ± .78</td>
<td>4.9 ± 1.37</td>
<td>2.7 ± .81</td>
</tr>
</tbody>
</table>

No significant differences were noted among the 3 groups regarding age, gender distribution, diabetes, time from transplant or differential cell counts.
### III.1.7. TABLE 2. BALF LEVELS OF LPO, GSSG AND AOP (n=37)

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS</th>
<th>BOS Op-1</th>
<th>BOS 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td><strong>BALF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected LPO</td>
<td>93.4 ± 19.7 (n=15)</td>
<td>88.5 ± 16.2 (n=12)</td>
<td>224.6 ± 24.9 (n=10)</td>
</tr>
<tr>
<td>Un-Corrected LPO</td>
<td>4.5 ± 1.23</td>
<td>7.8 ± 1.3</td>
<td>7.2 ± 1.0</td>
</tr>
<tr>
<td>Corrected GSSG</td>
<td>10.91 ± 2.26 (n=15)</td>
<td>6.79 ± 1.42 (n=12)</td>
<td>25.05 ± 5.24 (n=10)</td>
</tr>
<tr>
<td>Un-Corrected GSSG</td>
<td>.472 ± 0.04 (n=15)</td>
<td>.460 ± 0.06 (n=12)</td>
<td>.698 ± .127 (n=10)</td>
</tr>
<tr>
<td>Corrected AOP</td>
<td>1.28 ± .239 (n=14)</td>
<td>.871 ± .150 (n=12)</td>
<td>2.01 ± .441 (n=9)</td>
</tr>
<tr>
<td>Un-corrected AOP</td>
<td>.063 ± .009</td>
<td>.053 ± .011</td>
<td>.073 ± .014</td>
</tr>
<tr>
<td><strong>SERUM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOP (mM uric acid)</td>
<td>.180 ± .128 (n=19)</td>
<td>.264 ± .159 (n=16)</td>
<td>.188 ± .123 (n=11)</td>
</tr>
</tbody>
</table>

**Abbreviations:** **BALF:** Bronchoalveolar fluid; **MDA:** malondialdehyde; **LPO:** lipid peroxidation; **GSSG:** oxidized glutathione; **AOP:** Antioxidant potential.

**BALF LPO corrected:** p=0.001  
**BALF GSSG corrected:** p=0.001  
**BALF AOP corrected:** p=NS  
Significant difference among the three groups when corrected for BALF LPO and GSSG, not significant for corrected BALF AOP.
### III.1.7. Table 3. Plasma Antioxidant Levels

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS</th>
<th>BOS Op-1</th>
<th>BOS 2-3</th>
<th>Ref range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>µmol/l</td>
</tr>
<tr>
<td></td>
<td>(n=19)</td>
<td>(n=17)</td>
<td>(n=11)</td>
<td></td>
</tr>
<tr>
<td>( \gamma )-tocopherol (µmol/l)</td>
<td>2.92 ± .294</td>
<td>2.24 ± .328</td>
<td>2.52 ± .340</td>
<td>2-7</td>
</tr>
<tr>
<td>% pts below ( \gamma )-toco ref range</td>
<td>36</td>
<td>35</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-tocopherol (( \alpha )-toco) (µmol/l)</td>
<td>22.7 ± 2.91</td>
<td>23.4 ± 4.25</td>
<td>34.6 ± 5.84</td>
<td>12-46</td>
</tr>
<tr>
<td>( \alpha )-toco:cholesterol ratio</td>
<td>4.41 ± .602</td>
<td>4.52 ± .879</td>
<td>6.06 ± 1.15</td>
<td>&gt;2.2 µmol:mmol</td>
</tr>
<tr>
<td>♦ Retinol (µmol/l)</td>
<td>5.96 ± .572</td>
<td>5.39 ± .635</td>
<td>5.18 ± 1.01</td>
<td>1.50-3.0</td>
</tr>
<tr>
<td>% pts below Retinol ref range</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (µmol/l)</td>
<td>45.7 ± 3.77</td>
<td>66.4 ± 13.8</td>
<td>51.1 ± 6.51</td>
<td>23-84</td>
</tr>
<tr>
<td>% pts below Vit C ref range</td>
<td>19</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

♦ all trans retinol. Comparisons were done using one-way ANOVA and chi-square for categorical values. There were no statistically significant results among the three groups. \( P < 0.05 \) was considered statistically significant.
### III.1.7. Table 4. Macronutrient Intake

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS (n=22)</th>
<th>BOS Op-1 (n=16)</th>
<th>BOS 2-3 (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (Kcal)</strong></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td></td>
<td>(n=22)</td>
<td>(n=16)</td>
<td>(n=18)</td>
</tr>
<tr>
<td><strong>Kcal/kg</strong></td>
<td>1977 ± 724</td>
<td>1812 ± 620</td>
<td>1840 ± 544</td>
</tr>
<tr>
<td><strong>Protein (g/day)</strong></td>
<td>29.1 ± 12.3</td>
<td>25.5 ± 11.7</td>
<td>25.5 ± 7.7</td>
</tr>
<tr>
<td><strong>Protein g/kg</strong></td>
<td>81.8 ± 28.7</td>
<td>82.7 ± 20.8</td>
<td>80.7 ± 27.6</td>
</tr>
<tr>
<td><strong>Protein, % of energy</strong></td>
<td>1.19 ± .476</td>
<td>1.16 ± .479</td>
<td>1.12 ± .393</td>
</tr>
<tr>
<td><strong>Carbohydrate (CHO) (g/day)</strong></td>
<td>244.3 ± 88.3</td>
<td>224.2 ± 90.4</td>
<td>235.5 ± 80.2</td>
</tr>
<tr>
<td><strong>CHO g/kg</strong></td>
<td>3.56 ± 1.40</td>
<td>3.13 ± 1.48</td>
<td>3.28 ± 1.25</td>
</tr>
<tr>
<td><strong>CHO, % of energy</strong></td>
<td>49</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td><strong>Total Fat (g/d)</strong></td>
<td>77.12 ± 39.8</td>
<td>67.7 ± 29.8</td>
<td>65.2 ± 21.0</td>
</tr>
<tr>
<td><strong>Fat, % of energy</strong></td>
<td>35</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td><strong>SFA (g/d)</strong></td>
<td>27.2 ± 13.7</td>
<td>23.0 ± 9.1</td>
<td>23.5 ± 9.53</td>
</tr>
<tr>
<td><strong>PUFA (g/d)</strong></td>
<td>11.27 ± 6.20</td>
<td>13.7 ± 9.55</td>
<td>10.23 ± 4.03</td>
</tr>
<tr>
<td><strong>Fiber (g/day)</strong></td>
<td>16.6 ± 9.33</td>
<td>18.6 ± 10.09</td>
<td>18.9 ± 9.9</td>
</tr>
</tbody>
</table>

No Significant differences among the three patient groups
### III.1.7. **Table 5. Micronutrient Intake**

<table>
<thead>
<tr>
<th></th>
<th>Non-BOS</th>
<th>BOS OP-1</th>
<th>BOS 2-3</th>
<th>DRI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SEM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(n=22)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(n=16)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(n=18)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin A (VitA)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1712.8 ± 357</td>
<td>1627 ± 371</td>
<td>1163 ± 161</td>
<td>625 (m)</td>
</tr>
<tr>
<td></td>
<td>500 (f)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin A /1000 kcals</strong></td>
<td>938.9 ± 235</td>
<td>865.1 ± 145</td>
<td>702.2 ± 137</td>
<td></td>
</tr>
<tr>
<td><strong>% not meeting EAR</strong></td>
<td>27</td>
<td>12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin C (VitC)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/d)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>129.0 ± 23.1</td>
<td>109.5 ± 20.8</td>
<td>105.3 ± 13.81</td>
<td>75 (m)</td>
</tr>
<tr>
<td></td>
<td>60 (f)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin C /1000 kcals</strong></td>
<td>68.8 ± 11.1</td>
<td>65.3 ± 11.7</td>
<td>63.7 ± 10.4</td>
<td></td>
</tr>
<tr>
<td><strong>% not meeting EAR</strong></td>
<td>18</td>
<td>37</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin E (VitE)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.12 ± 2.38</td>
<td>6.85 ± 1.08</td>
<td>7.37 ± 1.11</td>
<td>15 (m)</td>
</tr>
<tr>
<td></td>
<td>15 (f)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Vitamin E /1000 kcals</strong></td>
<td>3.60 ± .598</td>
<td>3.66 ± .404</td>
<td>4.39 ± .872</td>
<td></td>
</tr>
<tr>
<td><strong>% not meeting EAR</strong></td>
<td>86</td>
<td>87</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations: ** DRI: Dietary Reference Intakes (2002); m: male, f: female; no significant differences among the three groups
III.1.8 DISCUSSION

Our results suggest that lung recipients with severe stage of BOS (BOS 2-3) were more oxidatively stressed compared to those patients with a milder stage of BOS (BOS Op-1) and non-BOS lung recipients. This was indicated by increased BALF levels of LPO and oxidized glutathione (GSSG).

Several studies have reported increased OxS in solid organ transplants (200,310,311) but only a few were studied in lung transplant patients. One prospective study (57) reported increased BALF MDA levels in lung recipients 2-weeks and 12 months post transplant compared to non-transplant controls (p<0.05). The authors observed no significant improvement in antioxidant status (serum or BALF) from 2 weeks to over one year following transplant, indicating that lung recipients remained oxidatively stressed. No association between OxS and time post transplantation was shown and there was no reported effect on graft function or BOS. Other authors also reported elevated BALF GSSG levels in BOS lung recipients (67) (247). Riise (247) reported 41% lower BALF GSH levels and an 850% increased level of BALF GSSG in BOS patients compared to non-BOS recipients. In a subsequent study with 13 non-BOS and 9 BOS lung recipients at 10-24 months post lung transplantation Behr (67) also found significantly reduced concentration of un-corrected BALF
glutathione in patients with BOS when compared with non-BOS (162.6 ± 20.1µM vs. 345.8 ± 57.1 µM) while oxidized glutathione was increased (13.9 ± 2.0% vs. 6.7 ± 1.2%).

When we analyzed the un-corrected values of BALF levels of LPO and GSSG, no statistical significance was observed between the different patient populations. However, interpretation of un-corrected values for soluble components of BALF must be viewed with caution due to variable returns on the volume instilled. For this reason, we corrected for the dilution effect by correcting for the level of urea. While no standardized procedures are validated in the lung transplant population, both albumin (312) and urea (252) have been used as dilution markers. We chose to use the BALF urea levels to correct for dilution as this method has been validated in patients with different lung diseases (313-315) and in lung transplant recipients (316,317). Moreover, this assay has also been shown to be sensitive (245).

OxS measurements in BALF did not show a linear relationship with BOS severity. Impaired epithelial injury and subsequent airway remodelling characterize BOS and perhaps lung recipients with milder stages of BOS have alveolar epithelial regeneration, resulting in increased antioxidants and antioxidant detoxification enzymes that are sufficient enough to diminish OxS occurring in the lung, resulting in lowered levels of LPO and GSSG. This would represent a similar
mechanism seen in Pulmonary Fibrosis patients (318). Although the precise mechanism of OxS in pulmonary fibrosis is as of yet unknown (319), the oxidant/antioxidant imbalance plays a crucial role (320).

Several factors contribute to OxS. Inflammation associated with BOS can be a major contributor due to increased production of ROS from neutrophils and macrophages. Although the severity of BOS in our subjects was appropriately characterized based on FEV1, there was no statistically significant difference in BALF macrophages and neutrophils among the three groups, suggesting that the patients were clinically stable at the time of the study.

Another contributing factor to OxS can be a weak antioxidant defense system. This was assessed by measuring BALF AOP and plasma. Measurement of AOP has been previously validated in different patient populations (235,237) and was found to be reliable and reproducible. It was also used in BALF of patients with lung injury (108,321). We found no significant difference among the 3 groups despite increases in OxS based on elevated LPO and GSSG. One possible explanation is that AOP is based on the reduction of Cu++ to Cu+ by the combined action of all antioxidants, like bilirubin, Vitamin E, Ascorbic Acid, Uric Acid and Glutathione. It is possible that the antioxidants in BALF had variable levels and the effect of low levels of certain antioxidants was compensated by higher levels of other
antioxidants resulting in a non-significant overall effect on AOP. Due to limited BALF volume, individual antioxidants could not be assessed. No other studies have been published in lung transplantation but one study in lung injury (245) showed a decrease in BALF AOP concentrations when compared with normal healthy controls.

Dietary intake can also influence OxS (322). To our knowledge, no studies assessing OxS in lung transplantation have been reported on nutritional intakes and plasma micronutrients. Our results show no significant differences in nutritional intakes or plasma antioxidant vitamin levels among groups.

In terms of plasma antioxidants, about 60% of our lung recipients had non-detectable plasma β-carotene, likely due to suboptimal intakes of β-carotene (323,324) based on our seven-day food records. β-Carotene functions as an antioxidant by quenching singlet \( \cdot \mathrm{O}_2 \) and scavenging other free radicals and therefore, low levels may contribute to OxS. Plasma \( \alpha \)-tocopherol and ascorbic acid were within reference range and not significantly different among groups.

In terms of nutritional intake, patients recorded adequate intakes of Vitamin A and Vitamin C based on Daily Recommended Intakes (DRI) and only about a third or less did not meet estimated average range (EAR) for these vitamins. However, despite normal plasma vitamin E levels, all patients failed to meet their DRI for
vitamin E and close to 80% did not meet their EAR. This has been also reported in the general population (325,326) and reflects low intake of nuts, seeds and oils. These foods may be avoided by the majority of lung recipients because of weight gain post transplantation (80).

In summary, increased OxS is evident in lung recipients with severe stage BOS. However, the antioxidant/oxidant balance following lung transplantation and the effect of this on the development of BOS is not known.

Our study has limitations. This was a cross-sectional design showing associations of OxS and BOS and not causation. The sample size was small, especially for BALF. This is a highly specialized and complex patient population prone to clinical complications. Therefore, enrolment presents some challenge. In addition, bronchoscopy is an invasive procedure and surveillance bronchoscopies are conducted only during the first 24 months post transplantation, unless medically indicated. Due to complex medical care and other ongoing studies, BALF was limited. Furthermore, our program conducts about 100 lung transplants per year and only 10% of patients develop BOS yearly, limiting our recruitment of BOS. The study design did not include healthy controls for ethical reasons because bronchoscopy is an invasive procedure associated with possible complications. In addition,
it is expensive and it is not an appropriate use of health care resources.

BOS represents increased airway epithelial injury and examining bronchial epithelium could have provided insight into the mechanism regarding the role of OxS and BOS. However this was beyond the scope of this project. A recent study (327) reported increased lung epithelia cellular senescence in lung recipients with and without BOS but no association between cellular senescence and BOS was found.

**III.1.9 Conclusions**

Patients with severe stage of BOS are more oxidatively stressed when compared to lung recipients with milder stages of BOS and non-BOS transplant patients. This was not associated with differences in nutritional intake or antioxidant levels in BALF or in plasma. Overall, lung transplant patients had low intakes of beta-carotene and vitamin E suggesting that this population may require dietary counseling or appropriate vitamin supplementation to correct low dietary intake. It is not clear whether this would reduce OxS and have a clinical impact on FEV\(_1\) and the development of BOS.
CHAPTER IV: OxS and HCV Liver Recipients

IV.1 ARTICLE: OxS AND NUTRITION IN HEPATITIS C LIVER RECIPIENTS, CONTROLS AND HCV NON-TRANSPLANT PATIENTS

By

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All authors report no conflict of interest.

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- Allied Health Research Day, Toronto, Ontario, Jan 29, 2009

This work was presented in a podium format at

- Multi-organ transplant rounds, Toronto Ontario, May 2009
IV.1.1 Abstract

**Background:** Hepatitis C Virus (HCV) is the most common indication for liver transplantation, but HCV recurrence is frequent after one year and associated with increased morbidity and mortality. OxS (OxS) is involved in the pathogenesis of HCV but little is known about its presence prior to disease recurrence.

**Aim:** To determine if at six months post transplant, HCV-LT without recurrence are oxidatively stressed.

**Methods:** Cross-sectional study with 33 HCV-LT, 12 controls (C) and 39 HCV non-transplant patients (HCV-NT). OxS was assessed by measuring liver lipid peroxidation (LPO) and antioxidant potential (AOP) (commercial kits). Plasma vitamin E, retinol (HPLC) and Vitamin C (spectrophotometry) were assessed. Anthropometry and three-day food records were collected. Analysis by Kruskal-Wallis and expressed as Mean ± SEM.

**Results:** Mean age, BMI and gender were not different among the three groups. Waist Hip Ratio (WHR) was higher in both HCV-LT and HCV-NT when compared to controls. HCV-LT had higher hepatic LPO (µmol MDA/g tissue) versus controls: 1.4 ± 0.20 vs. 0.54 ± 0.10 (p=0.010) and when compared to HCV-NT: 0.98 ± 0.17 (p=0.030). No significant differences were seen among the three groups regarding hepatic AOP. However, lower plasma AOP (µmols UEA) were found in
HCV-LT: 0.07 ± 0.008 versus Controls: 0.17 ± .040, (p=0.021) and HCV-NT: 0.08 ± 0.009 (p=0.015) versus controls. Plasma gamma-tocopherol was higher in HCV-LT and HCV-NT compared to C (p=0.001). Antioxidant intake was similar except lower vitamin A intake, in HCV-LT compared to the other two groups (p=0.001).

**Conclusions:** HCV-LT recipients, without disease recurrence, are oxidatively stressed when compared to controls and HCV-NT. Future research is needed to determine the impact of this increased OxS on HCV disease recurrence.
**IV.1.2 INTRODUCTION**

Approximately 170 million people worldwide are infected with Hepatitis C virus (HCV) (120). This represents a global health problem of pandemic proportions (121). HCV is a leading cause of chronic liver disease and the most common indication worldwide for liver transplantation. Reinfection of the virus post transplant is universal, however, HCV disease recurrence is highly unpredictable and the progression to cirrhosis is accelerated compared to immunocompetent HCV patients (231).

OxS plays a role in the pathogenesis of HCV although the exact mechanism is unknown. Upon HCV infection, increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in neutrophils by the non-structural protein 3 (NS3) HCV core protein (328,329). In addition, increased ROS/RNS are produced in hepatocytes, through the release of inflammatory cytokines such as TNFα and IL-1β (157). Oxidative injury occurs when the ROS/RNS overwhelm the antioxidant defense system (330). Production of ROS/RNS can be influenced by many factors such as chronic inflammation, iron overload, drugs, and direct effects of Hepatitis C viral proteins and, in the context of transplantation, ischemia-reperfusion injury, and rejection (331).
addition liver steatosis may increase OxS by increasing lipid peroxidation (332). Liver steatosis can be associated with obesity (333), particularly abdominal obesity, measured by waist-to-hip ratio (334) and also with genotype 3 (335). The level of OxS is also influenced by the antioxidant system, which is composed of antioxidant enzymes and micronutrients. This in turn can be influenced by the chronicity of ROS production and nutritional intake (218,322).

Currently there is very limited data on OxS in HCV liver transplant and it is not clear whether OxS is present in this patient population and whether it contributes to HCV disease recurrence. Therefore, the aim of this study was to determine whether OxS is present in HCV-LT, before disease recurrence, and compares the results to a control group and HCV-NT patients.

**IV.1.3 MATERIAL AND METHODS**

**IV.1.3.1 Study Design**
Between February 2007 to February 2008, HCV-LT who were attending the UHN liver transplant clinic and were about four to six months post transplant were consecutively recruited. This was a cross-sectional study including three groups.

**IV.1.3.2 Subjects**

Group 1: HCV Liver transplant recipients (HCV-LT). Fifty-five HCV-LT patients attended clinic, 10 patients were not recruited (7
declined and 3 patients failed to attend their scheduled biopsy), and 45 patients were enrolled. HCV-LT recipients were excluded if they had evidence of recurrence defined as a Metavir score (histological scoring system) >1; were re-transplanted; had acute rejection at the time of entry into the study or consumed alcohol. For the HCV-LT, HCV disease recurrence was defined based on a liver biopsy indicating only mild fibrosis (injury) in the absence of other causes such as rejection, other viral infections or drug toxicity. Twelve patients were found to have Metavir score > 1. We therefore report on 33 HCV liver recipients.

Group 2: Control subjects: The control group consisted of patients referred to the hepatology clinic for mildly elevated liver enzymes with an alcohol consumption of less than 20 grams/day. They had no evidence of liver disease on subsequent liver biopsies.

Group 3: HCV non-transplant patients (HCV-NT): Patients were recruited from the hepatology clinic; they were not on any antiviral medications and had alcohol consumption of less than 20g/day.

**IV.1.3.3 Methods**

Consented patients meeting the study criteria had their blood collected after a 12-h fast for the various measurements. In addition, for the HCV-LT group, pre-transplant viral load was obtained from the medical
record. The viral load was assessed using the Roche PCR method (336).

HCV-LT had surveillance biopsies scheduled at approximately six-month post transplant. Necroinflammation activity and fibrosis stage were scored according to METAVIR (284).

Height, weight, waist and hip were measured to calculate BMI and waist to hip ratio. Food records were kept for three consecutive days to measure intake of macro and micronutrients. Subjects were instructed to eat their regular meals and itemize the food using a standard portion size pamphlet (food portion visual 2.0; Nutrition Consulting Enterprises, Framingham, MA). The data were analyzed using West-Can Diet Analysis Plus Version 8.0; 2005. The Estimated Average Requirement (EAR) for estimating prevalence of inadequate intake was used. The distribution was determined by examining proportion above and below EAR (276).

The primary variable of interest was liver levels of Lipid Peroxides (LPO). The secondary variables included Liver Antioxidant Potential (AOP), plasma AOP, antioxidant vitamins C, E and carotenoids. Nutritional status, intakes of polyunsaturated fatty acids (PUFA) and antioxidant nutrients were also assessed.

A portion of liver tissue was frozen in liquid nitrogen within 15 minutes of collection and stored at -80°C until analysis. Liver tissue
was weighed and homogenized in ice-cold 20 mM PBS buffer, pH 7.3 with 5mM butylated-hydroxy-toluene to avoid ex-vivo oxidation. The suspension was centrifuged and supernatant was fractionated for analysis of LPO and AOP.

Liver LPO was measured using a commercially available kit (LPO 586, Oxis International, Portland, USA), which measures free malondialdehyde (MDA) and 4-hydroxyalkenals. This method is based on a reaction of N-methyl-2-phenylindole with MDA and 4-hydroxyalkenals at 45 °C and read at absorbance of 586 nm.

Liver AOP was assessed using a commercially available kit, Bioxytech AOP-490 kit (Oxis Research, a division of OXIS Health Products, Inc, Portland, Oregon, USA) (321). The assay is based upon the reduction of Cu$$^{++}$$ to Cu$$^{+}$$ by the combined action of all antioxidants such as bilirubin, albumin, Vitamin E, Ascorbic Acid, Uric Acid and Glutathione present in the sample. A chromogenic reagent, Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu$$^{+}$$ which has a maximum absorbance at 490 nm (337).

Blood was collected in EDTA-containing vacutainers and centrifuged at 910 x g; 10 min to separate plasma. Plasma AOP was assessed using a commercially available kit, Bioxytech AOP-490 kit (details described above).
For vitamin C measurement plasma was stabilized immediately with 100 g HPO₃ (meta-phosphoric acid)/L (0.5 ml of plasma plus 0.5 ml of HPO₃)(1:1) and was stored at −80°C for later analysis. Samples were analyzed for total biologically active vitamin C by spectrophotometry, using 2,4,-dinitro-phenylhydrazine as the chromogen (259,306).

Alpha-and gamma-tocopherol, and retinol were analyzed by HPLC and fluorescence spectrophotometry. Lipids were extracted from 200 µl of plasma, using beta-hydroxy-toluene (BHT, 35 mg in 100 ml) containing HPLC-grade n-hexane (1 ml) and HPLC-grade ethanol (400 µl). Peaks were separated using a reverse-phased C18 column at flow rate of 1.0 ml/min. A Varian programmable multi-wavelength detector was used to detect tocopherols, retinol and carotenoids at 292, 325, and 450 nm respectively (269).

**IV.1.4 Ethics Approval**

This study was performed according to the guidelines of the 1975 Declaration of Helsinki and was approved by the Research Ethics Board, University of Health Network, Toronto, Ontario, Canada. Informed consent for each participant was obtained (Appendix 4).

**IV.1.5 Statistical Analysis**

Data are expressed as Mean ± SEM. For each variable, a comparison of means was completed. If the variable displayed normal
distribution, one-way ANOVA was utilized and Tukey’s post hoc test was conducted. If data were not normally distributed, the equivalent non-parametric Kruskal-Wallis test was used. Chi-square test was used for categorical variables. SPSS 16.0, was used for analysis, and statistical significance was defined as p<0.05.

**IV.1.6 Results**

The demographic profile of the three patient groups is described in Table 1. Age, gender distribution, and proportion of patients with diabetes were similar among the three groups. BMI and percent body fat was also similar among the three groups. However, HCV-LT (.964 ± .025) and HCV-NT patients (.970 ± .015) had significantly higher WHR when compared to controls .882 ± .023; (p=0.016) and (p=0.006;) respectively (**IV.1.6.Table 1**).

Seventy-five percent of the HCV-LT had biopsy-proven minimal to mild inflammation compared to 1% in controls (p=0.001) and 10% in HCV-NT (p=0.001).

Hepatic levels of LPO were significantly higher in HCV-LT (µmolMDA/g tissue) (1.4 ± .20); when compared to Controls (.54 ± .10); (p=0.010) and when compared to HCV-NT (.98 ± .17); (p=0.030) (**IV.1.6.Figure 1a**), and HCV-NT had higher hepatic LPO when compared to controls (p=.015). There were no significant differences in hepatic AOP levels (µmols uric acid/g tissue) among the
three groups: HCV-LT: 19.0 ± 2.29; Controls: 29.5 ± 5.9; HCV-NT: 18.5 ± 2.1 (IV.1.6.Figure 1b).

Plasma AOP (µmols uric acid) was lower in HCV-LT (0.07 ± .008) when compared to Controls (.17 ± .040); p=0.021 but not when compared to HCV-NT (.08 ± .009) (IV.1.6.Figure 1c). As well, lower plasma AOP were seen in HCV-NT patients when compared to controls (p=0.015).

IV.1.6.Table 2 describes the plasma antioxidant levels in the three patient groups. Although plasma α-tocopherol was similar among the three groups, gamma-tocopherol (µmol/l) was significantly higher in both the HCV-LT and HCV-NT patients versus controls. All other plasma levels were not significantly different among the three groups. There was no significant difference in caloric intake or intakes of protein, fat or carbohydrate, among the three groups (IV.1.6.Table 3). Micronutrient intake was also similar except for vitamin A. HCV-LT consumed significantly less Vitamin A (µg/d), when compared to Controls and when compared to HCV-NT patients. The EAR was not met for Vitamin E in all three patient groups (IV.1.6.Table 4)

No correlations were found between our primary variable of interest: liver LPO and other variables such as liver AOP, liver pathology and/or inflammation or intake of micronutrients.
### IV.1.6. Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>HCV-LT</th>
<th>Control</th>
<th>HCV-NT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM (n=33)</td>
<td>Mean ± SEM (n=12)</td>
<td>Mean ± SEM (n=39)</td>
</tr>
<tr>
<td><strong>AGE (YEARS)</strong></td>
<td>55.0 ± 1.0</td>
<td>50.6 ± 1.7</td>
<td>52.0 ± 1.0</td>
</tr>
<tr>
<td><strong>GENDER (%F/%M)</strong></td>
<td>16/84m</td>
<td>40/60</td>
<td>35/65</td>
</tr>
<tr>
<td><strong>% DIABETES MELLITUS</strong></td>
<td>40</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27.2 ± 0.579</td>
<td>28.0 ± 1.55</td>
<td>26.5 ± 1.10</td>
</tr>
<tr>
<td><strong>% PT BMI &gt;27 kg/m²</strong></td>
<td>50</td>
<td>41</td>
<td>44</td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>.964 ± .025</td>
<td>.882 ± .023</td>
<td>.970 ± .015</td>
</tr>
<tr>
<td><strong>SERUM ALT (U/L)</strong></td>
<td>70.7 ± 9.9</td>
<td>60.8 ± 11</td>
<td>61.6 ± 6.4</td>
</tr>
<tr>
<td><strong>% MINIMAL-MILD INFLAMMATION</strong></td>
<td>75</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><strong>VIRAL LOAD (IU/mL)</strong></td>
<td>5.05 x10⁶ ± 5.12x10⁶</td>
<td>N/a</td>
<td>1.67 x10⁶ ± 3.02x10⁶</td>
</tr>
</tbody>
</table>

**ABBREVIATIONS:**

- **HCV-LT:** HCV liver transplant patients; **Controls:** referred for mildly elevated liver enzymes but normal or minimal findings on liver biopsy; **HCV-NT:** HCV non-transplant patients.
- **BMI:** body mass index; **WHR:** waist-to-hip ratio; **HCV-LT vs Control:** p=0.016; **HCV-NT vs Control:** p=0.006

Biopsy proven minimal to mild inflammation: % mild inflammation HCV-LT versus controls and versus HCV-NT: p=0.001 for both.

Viral load levels are pre-transplant for HCV-LT; post-transplant viral loads not conducted unless patients are on antiviral medication. No other significant differences among the groups.
OxS and Antioxidant Measurements in Liver and Plasma

IV.1.6. Figure 1a. Liver LPO (Mean ± SEM)

Figure 1a. Liver LPO MEAN ± SEM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean ± SEM (umoles MDA/g liver)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-LT</td>
<td>1.4 ± 0.20</td>
<td>0.030</td>
</tr>
<tr>
<td>Controls</td>
<td>0.54 ± 0.10</td>
<td>0.010</td>
</tr>
<tr>
<td>HCV-NT</td>
<td>0.98 ± 0.17</td>
<td>0.015</td>
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</table>
IV.1.6. Figure 1b. Liver AOP

Figure 1b. LIVER AOP

<table>
<thead>
<tr>
<th></th>
<th>HCV-LT</th>
<th>CONTROLS</th>
<th>HCV-NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>micromoles uric acid/g liver</td>
<td>19.0 ± 2.29</td>
<td>29.5 ± 5.9</td>
<td>18.5 ± 2.1</td>
</tr>
</tbody>
</table>

p = NS
IV.1.6. Figure 1c. Plasma AOP

PLASMA AOP MEAN ± SEM

<table>
<thead>
<tr>
<th></th>
<th>HCV-LT</th>
<th>CONTROLS</th>
<th>HCV-NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>umols uric acid</td>
<td>0.07 ± .008</td>
<td>0.17 ± .040</td>
<td>0.08 ± .009</td>
</tr>
</tbody>
</table>

p = 0.021
p = 0.015
## IV.1.6. Table 2. Plasma Antioxidants

<table>
<thead>
<tr>
<th></th>
<th>HCV-LT</th>
<th>Control</th>
<th>HCV-NT</th>
<th>Ref range</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>µmol/l</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=12)</td>
<td>(n=20)</td>
<td></td>
</tr>
<tr>
<td>γ-tocopherol µmol/l</td>
<td>$3.37 \pm .28^a$</td>
<td>$1.84 \pm .289$</td>
<td>$3.08 \pm .315$</td>
<td>2-7</td>
</tr>
<tr>
<td>% below γ-toco ref range</td>
<td>36</td>
<td>35</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol(α-toco) µmol/l</td>
<td>$22.9 \pm 1.56$</td>
<td>$23.0 \pm 4.30$</td>
<td>$25.2 \pm 2.34$</td>
<td>12-46</td>
</tr>
<tr>
<td>♦Retinol µmol/l</td>
<td>$3.36 \pm .326$</td>
<td>$3.56 \pm .355$</td>
<td>$3.17 \pm .261$</td>
<td>1.50-3.0</td>
</tr>
<tr>
<td>% below Retinol ref range</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vitamin C µmol/l</td>
<td>$57.7 \pm 5.33$</td>
<td>$85.9 \pm 18.7$</td>
<td>$52.4 \pm 5.66$</td>
<td>23-84</td>
</tr>
<tr>
<td>% below Vit C ref range</td>
<td>19</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
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</table>

♦all trans retinol

**HCV-LT:** Gamma-tocopherol compared to Controls: p=0.016

**HCV-NT:** Gamma-tocopherol compared to Controls: p=0.026

**HCV-LT:** Gamma-tocopherol compared to HCV-NT, p=NS

No other significance was noted.
### IV.1.6. Table 3. Macronutrient Intake

<table>
<thead>
<tr>
<th></th>
<th>HCV-LT (Mean ± SEM, n=22)</th>
<th>Control (Mean ± SEM, n=12)</th>
<th>HCV-NT (Mean ± SEM, n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (Kcal)</strong></td>
<td>1809 ± 92</td>
<td>2227 ± 232</td>
<td>1820 ± 151</td>
</tr>
<tr>
<td><strong>Protein (g/day)</strong></td>
<td>80.2 ± 4.1</td>
<td>94.3 ± 10.9</td>
<td>81.3 ± 7.4</td>
</tr>
<tr>
<td>% of energy Protein</td>
<td>18</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td><strong>Carbohydrate (g/day)</strong></td>
<td>203 ± 12.9</td>
<td>301 ± 40.3</td>
<td>219 ± 18.8</td>
</tr>
<tr>
<td>% of energy</td>
<td>45</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td><strong>Total Fat (g/day)</strong></td>
<td>72.3 ± 4.5</td>
<td>72.9 ± 9.0</td>
<td>72.2 ± 9.8</td>
</tr>
<tr>
<td>% of energy Fat</td>
<td>37</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td><strong>PUFA (g/day)</strong></td>
<td>9.65 ± 1.03</td>
<td>15.8 ± 2.2</td>
<td>12.7 ± 2.05</td>
</tr>
<tr>
<td><strong>Fiber (g/day)</strong></td>
<td>16.9 ± 1.02</td>
<td>27.2 ± 4.4</td>
<td>22.7 ± 3.2</td>
</tr>
</tbody>
</table>

One-way Anova used to compare groups. The acceptable range of macronutrient composition in the diet for adults is 20-35% fat, 45-65% carbohydrates and 10-35% protein. DRIs: Food and Nutrition Board. Institute of Science, 2002

EER: Calories: Recommended Fiber intake: Males: 38 g/d; females 25
g/d Fat: 30% of calories
No significant differences among the groups.
### IV.1.6. Table 4. Micronutrient Intake

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>HCV-LT Mean ± SEM (n=22)</th>
<th>Controls Mean ± SEM (n=12)</th>
<th>HCV-NT Mean ± SEM (n=18)</th>
<th>DRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (VitA) (µg/d)</td>
<td>797 ± 83.1</td>
<td>2499 ± 764</td>
<td>1979 ± 279</td>
<td>625 (m) 500 (f)</td>
</tr>
<tr>
<td>% not meeting EAR</td>
<td>50</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (VitC) (mg/d)</td>
<td>85.9 ± 10.41</td>
<td>138.3 ± 27.5</td>
<td>103.9 ± 21.9</td>
<td>75 (m) 60 (f)</td>
</tr>
<tr>
<td>% not meeting EAR</td>
<td>60</td>
<td>25</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (VitE) (mg/d)</td>
<td>5.29 ± .676</td>
<td>7.54 ± .817</td>
<td>4.31 ± .658</td>
<td>15 (m) 15 (f)</td>
</tr>
<tr>
<td>% not meeting EAR</td>
<td>97</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Vitamin A intakes:
- HCV-LT vs Control: p=0.001;
- HCV-LT vs HCV-NT p=0.002;
- HCV-NT vs Control p=NS

EAR: Estimated Average Requirements, Food and Nutrition Board, Institute of Medicine (2002)
IV.1.7 DISCUSSION

This is the first study investigating OxS in the liver of HCV-LT, six months post-transplant. In addition, this study assessed plasma micronutrient antioxidants, nutritional intake and anthropometry. We report increased hepatic OxS in HCV-LT compared to controls and HCV-NT patients despite overall similar nutritional and antioxidant measurements.

Only two other studies reported on OxS, measured in urine or plasma, in the general patient population post liver transplant. One prospective study (201) indicated that OxS was evident in post transplant recipients one year after surgery. Fifty pre and post-liver transplants (25 HCV negative, 25 HCV positive) were compared to 30 healthy controls. OxS was determined by measuring urinary dinor-dihydro iPF$_{2\alpha}$-111 levels (a urinary LPO marker). Significantly higher urinary levels of this LPO marker were reported in pre and post transplant recipients compared to healthy controls. No measurements were performed in the liver and HCV positive and negative patients were not analyzed separately. No associations between OxS and acute cellular rejection, organ failure, or infection of the allograft by HCV were found. No measurements of the antioxidant system or nutritional intake were performed. Similarly, in a cross sectional study (200), increased plasma thiobarbituric acid-reactant substances (TBARS)
(representing the total lipid peroxidation products) and low plasma α-tocopherol were reported in 20 cirrhotic patients and 22 post LT recipients (who were at least six-months post transplant), compared to thirty healthy volunteers. Following transplant, decreased plasma TBARS and increased α-tocopherol levels were noted, indicating some improvement in OxS, however, these values did not reach healthy control levels. No correlations were noted between OxS parameters and liver function tests, disease recurrence or rejection episodes. No other antioxidant micronutrients were measured and dietary intake was not performed. The results of these two studies indicate that OxS persists post transplant in both HCV positive and negative recipients, based on peripheral measurements (urine and plasma). However, OxS was not specifically studied in HCV-LT at six months post-transplant. Furthermore, no liver measurements were performed and no associations were made with liver pathology, viral load, antioxidants and nutritional status.

A weak antioxidant defense system can also contribute to OxS, and this was determined in our study by measuring hepatic and plasma levels of AOP. We found significantly lower plasma AOP levels in HCV-LT and in HCV-NT patients when compared to controls. Very little is known about the antioxidant status in HCV-LT. Only one study in HCV non-transplant liver patients reported on the total
antioxidant capacity (TAOC) with no difference in HCV immunocompetent patients when compared to controls (168).

Altered levels of single plasma antioxidants can also contribute to OxS. There was no significant difference among groups regarding antioxidant vitamins, except for elevated plasma gamma-tocopherol levels in both HCV-LT and HCV-NT when compared to controls. To our knowledge, this is the first report of elevated \( \gamma \)-tocopherol levels in HCV patients and in HCV-LT. Vitamin E exists in eight isomeric forms (338) (339) and the majority of vitamin E containing foods are rich sources of \( \gamma \)-tocopherol (340). Alpha-tocopherol is the major chain-breaking lipid soluble antioxidant preventing LPO (341,342). \( \gamma \)-tocopherol functions as an antioxidant by scavenging RNS and although it is not as efficient an antioxidant as \( \alpha \)-tocopherol, there is emerging evidence that it may play a more important role than once thought (343,344). The reason for these increased plasma \( \gamma \)-tocopherol levels are not clear, however, \( \gamma \)-tocopherol is metabolized by the Cytochrome P450 system (345), which may be impaired in HCV liver patients. HCV represents an inflammatory process and the CYP450 activity is inhibited by interleukins and other pro-inflammatory cytokines leading to a decreased degradation of \( \gamma \)-tocopherol and increased plasma levels (346). Two other studies have reported increased plasma \( \gamma \)-tocopherol levels in chronic inflammatory
conditions. Significantly elevated levels have been reported in smokers versus non-smokers (347) and similarly in hemodialysis patients compared to healthy controls (348). These studies support our results regarding elevated $\gamma$-tocopherol in the context of HCV-LT.

Low intakes of antioxidants may also contribute to increase OxS (322). Our results indicate that HCV-LT consumed sub-optimal intakes of vitamin A when compared to Controls and HCV-NT. Despite this, no difference was seen in plasma levels. As well, despite plasma vitamin E levels within normal range, all patients failed to meet their DRI for vitamin E and the majority of patients did not meet their EAR. HCV-LT consumed sub-optimal intakes of antioxidant micronutrients and have biopsy-proven increased OxS and inflammation, suggesting that they may benefit from increased antioxidant intake either from dietary sources or supplementation.

Cyclosporine A (CsA) and Tacrolimus (TAC) are potent immunosuppressive agents used for the prevention of graft rejection. Both drugs are metabolized through the cytochrome P450 system and are associated with intracellular depletion of reduced glutathione and increased lipid peroxidation and both drugs possess pro-oxidant activity (207). CsA has been shown to increase the production of ROS in rat microsomes (208), and in human microsomes (349) leading to increased LPO. This was confirmed in another animal study (210).
TAC also increases the production of ROS (211). Since ROS/RNS react with macromolecules, especially lipids, causing increased lipid peroxidation it is tempting to speculate that HCV-LT have increased LPO compared to HCV non-transplant patients due to their immunosuppressive medications.

Increased liver iron concentrations also promote increased OxS (161) and this has been previously documented in non-transplant HCV patients (162). However, iron stains performed on biopsy specimens were negative in HCV-LT suggesting that altered iron status was not a cause for increased OxS.

The inflammatory process associated with obesity can also increase OxS (350). BMI was not significantly different among the three patient groups but WHR (a measure of abdominal obesity) was significantly higher in both HCV groups compared to controls. This could have contributed to the increased OxS seen in HCV-LT.

We also noted on biopsy, that the proportion of patients with mild inflammation was increased in HCV-LT when compared to controls and HCV-NT. Inflammation may contribute to the increased OxS observed in HCV-LT and this, in turn, can activate stellate cells and induce fibrogenesis (351,352). Evidence suggests that early increased inflammation in HCV recipients is associated with rapid development of
fibrosis (353). Increased OxS associated with chronic inflammation has been previously reported in non-transplant HCV patients (354).

There are limitations to this study. This was a cross sectional study and thus cause and effect inferences are not possible. Furthermore, our ‘control’ group was initially referred for mildly elevated liver enzymes, which was the indication for liver biopsy. It would have been preferable to perform a liver biopsy on healthy volunteers, but this is ethically not feasible. However, we presume that since we already found a significant difference in the main variable of interest among the HCV groups and our control group with mildly elevated liver enzymes, this difference would have been greater if compared to healthy volunteers. We did not study a non-HCV liver transplant group because, as per protocol, these non-HCV-LT do not have routine liver biopsies post transplant. This is a limitation as it is possible that the OxS observed in the liver of the HCV-LT may also be present in non-HCV recipients and may be caused, in part, by the immunosuppressive medications. No previous studies have assessed OxS in the liver of HCV or non-HCV patients six months post transplant and therefore we cannot determine whether the elevated OxS is related to transplant itself or transplant related factors such as drugs.

IV.1.8 CONCLUSIONS
In conclusion, HCV-LT without HCV disease recurrence are more oxidatively stressed when compared to controls and HCV-NT patients. This was also associated with significant lower plasma antioxidant potential, low intakes of vitamin A but elevated plasma \( \gamma \)-tocopherol. Antioxidant vitamin levels and intake were overall similar but all three patient groups had low intakes of vitamin E. It would be of interest in future studies to determine whether the presence and degree of OxS in HCV-LT can predispose to disease recurrence and whether antioxidant supplementation may reduce OxS and affect this outcome.
CHAPTER V: HEPATIC LPO AND HCV LIVER RECIPIENTS

V.1 ARTICLE: HEPATIC LIPID PEROXIDATION AND ANTIOXIDANT MICRONUTRIENTS IN HCV LIVER RECIPIENTS WITH AND WITHOUT DISEASE RECURRENCE.

By

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- Canadian Society of Clinical Nutrition, Quebec City, Quebec, May 19, 2009
- Canadian Foundation for Dietetic Research, Annual Dietitians of Canada conference, PEI, June 2009
**V.1.1 Abstract**

*Introduction:* Hepatitis C virus (HCV) re-infection following liver transplantation is universal and 10-30% progress to cirrhosis. Several risk factors are associated with progression. OxS (OxS) may be involved as it plays a role in the pathogenesis of HCV.

*Aims:* To determine if HCV liver transplant patients (HCV-LT) with disease recurrence are more oxidatively stressed compared to those with no recurrence.

*Methods:* This study involved a cross-sectional and prospective design. Measurements were performed at 12 months (cross-sectional) and in a subgroup of patients (prospective), at six-months post-transplant. Liver lipid peroxidation (LPO), antioxidant potential (AOP), plasma Vitamin E, retinol and Vitamin C were measured. Demography, pre-transplant viral load, anthropometry and three-day food records were also obtained. Data were log-transformed and analyzed by independent t-test; chi-square for categorical; Pearson correlation and multivariate regression analysis.

*Results:* Thirty-seven patients were evaluated at 12 months post transplant: 21 with no recurrence and 16 with recurrence. HCV-LT with recurrence had higher liver LPO (μmol malondialdehyde (MDA)/gram of liver tissue) when compared to those with no recurrence (1.66 ± .279 vs 0.878 ± .128, p=0.015). A significant relationship was found between liver LPO and HCV disease recurrence and this association remained after adjusting for pre-transplant viral load and donor age. Six patients with recurrence and 11 with no recurrence also had measurements performed at six-months post transplant. Those with recurrence at 12 months had significantly higher hepatic LPO at six-months compared to those with no recurrence (1.86 ± 0.619 vs 0.746 ± 0.143, p=0.038). No other significant differences between the two groups were noted. Neither group was meeting Dietary Reference Intake (DRI) for Vitamin E.

*Conclusions:* HCV-LT patients with disease recurrence are more oxidatively stressed at 6 and 12 months post transplant when compared to those with no recurrence. Taking into account viral load and donor age, OxS was independently associated with recurrence. More research is needed to confirm this association and to determine if antioxidants would be of benefit.
**V.1.2 Introduction**

HCV liver disease is the most common indication for liver transplantation worldwide (120). Unfortunately, 100% of the transplant recipients are reinfected with the virus (130) and 50% of the patients develop histological evidence of HCV disease recurrence by one year (131). Ten to thirty percent of the patients, progress to cirrhosis by 5-years post transplant. Therefore, HCV-LT have lower survival rates than non-HCV liver recipients.

Established risk factors associated with the progression of HCV disease recurrence include pre-transplant viral load, early post-transplant viral load, steroid bolus for acute rejection episodes, CMV infection and donor age (202-206,355).

OxS has been shown to play a role in the pathogenesis of HCV disease in the pre-transplant population (354). OxS occurs when prooxidants overwhelm the antioxidant defense system and this occurs in inflammatory conditions such as infection. General risk factors associated with increased OxS include: obesity (71,72); diabetes (356,357) hepatic steatosis (358, 359) and type of immunosuppression (207).

Studies have shown that plasma (200) and urinary levels (201) of lipid peroxidation (LPO) end products such as malondialdehyde (MDA) and PGF\(_{2-\alpha}\) are increased in HCV-LT patients up to one-year
post transplant, indicating that HCV-LT patients may be oxidatively stressed. However, no studies have measured OxS parameters in the liver or assessed intake and plasma levels of antioxidants in HCV-LT patients. In addition, no studies have compared HCV recurrence versus no recurrence nor has any study been completed to determine whether OxS in the liver can predispose to disease recurrence. This may be of interest since OxS contributes to the pathogenesis of HCV, including inflammation and fibrogenesis (353). OxS, if present can be reduced by antioxidant supplementation. Since there is no ideal treatment for HCV to halt the progression of disease recurrence, determining if OxS plays a role in disease recurrence may be beneficial.

The primary aim of this study was to determine if HCV-LT patients with recurrence at 12 months are more oxidatively stressed compared to those with no disease recurrence. A secondary aim was in a sub-group of patients, to determine if those with recurrence were also more oxidatively stressed at six-months.

V.1.3 MATERIAL AND METHODS

V.1.3.1 Study Design

Between February 2007 and February 2008, patients attending UHN ambulatory liver clinic who were approximately four to six months or 10-12 months post transplant were approached for the study.
V.1.3.2 Subjects

Those meeting the study criteria were enrolled. After signing the informed consent, demographics, such as age, weight, BMI, medical and drug history were extracted from our transplant database (Organ Transplant Tracking Record, Kenyon Hicks, Omaha, NE). Subjects were followed at 6 and 12 months, with surveillance liver biopsy to assess activity and fibrosis level. Patients with biopsy results indicating METAVIR (284) which included fibrosis score 1 or more were classified as HCV disease recurrence.

The study criteria include stable HCV-LT who were between 18-65 years of age. Liver recipients who had been re-transplanted, had multiple organ transplants, or who had acute rejection at the time of entry into the study were excluded.

The primary variable of interest was Liver Lipid Peroxidation (LPO) and secondary variables were Liver Antioxidant Potential (AOP) and plasma AOP, antioxidant vitamins C, E and carotenoids. In addition, nutritional parameters were also assessed using three-day food records to measure macronutrient intake including polyunsaturated fatty-acids (PUFA) and micronutrient antioxidant intakes and anthropometry.

V.1.3.3 Methods

Necroinflammation activity and fibrosis (Appendix 5), were scored according to METAVIR (284). All biopsies were read by one of two experienced liver pathologists.

In addition, a portion of the liver biopsy was saved for assessment of OxS. The patients also had blood drawn at their regular clinic visit for measurements of plasma AOP and antioxidants such as Vitamins C, E and carotenoids. Consecutive three-day food records were obtained
from each patient at the time of scheduled clinic blood work, to
determine macro and micronutrient intakes. Anthropometric data such
as Body Mass Index (BMI), Bioelectrical Impedance (BIA), and Waist-
to-hip ratio (WHR) were calculated.

For the liver measurements, liver tissue was frozen in liquid
nitrogen within 15 minutes of collection and stored at -80°C. For the
analysis, liver samples were weighed and homogenized in ice-cold 20
mM PBS buffer, pH 7.3 with 5mM butylated-hydroxy-toluene to avoid
ex-vivo oxidation. Liver LPO was measured using a commercially
available kit (LPO 586, Oxis International, Portland, USA), which
measures free malondialdehyde (MDA) and 4-hydroxyalkenals. This
method is based on a reaction of N-methyl-2-phenylindole with MDA
and 4-hydroxyalkenals at 45 °C and read at absorbance of 586 nm.
Liver AOP was assessed using a commercially available kit, Bioxytech
AOP-490 kit (Oxis Research, a division of OXIS Health Products, Inc,
Portland, Oregon, USA) (321). The assay is based upon the reduction
of Cu²⁺ to Cu⁺ by the combined action of all antioxidants such as
bilirubin, albumin, Vitamin E, Ascorbic Acid, Uric Acid and Glutathione
present in the sample (337).

For plasma antioxidant measurements, the blood was collected
in EDTA-containing vacutainers and centrifuged at 910 x g; 10 min to
separate plasma. Plasma AOP was assessed using a commercially
available kit, Bioxytech AOP-490 kit (as above). For vitamin C measurement plasma was stabilized immediately with 100g HPO$_3$ (meta-phosphoric acid)/L (0.5 ml of plasma plus 0.5 ml of HPO$_3$) (1:1) and was stored at –80° until analysis. At the time of analysis, the total biologically active vitamin C was analyzed by spectrophotometry (306). Alpha- and gamma-tocopherol, and retinol were analyzed by HPLC and fluorescence spectrophotometry (269). Lipids were extracted from 200 µl of plasma; peaks were separated using a reverse-phased C18 column. A Varian programmable multi-wavelength detector was used to detect tocopherols, retinol and carotenoids at 292, 325, and 450 nm respectively (269). These methods have been validated in a variety of disease conditions (227, 235-237).

V.1.4 Ethics approval

This study was performed according to the guidelines of the 1975 Declaration of Helsinki and was approved by the Research Ethics Board, University of Health Network, Toronto, Ontario, Canada.

V.1.5 Statistical analysis

Variables with a skewed distribution were log-transformed for analyses and independent t-tests were conducted. Pearson correlation analysis was conducted and chi-square for categorical variables. Donor age was tested as a continuous and categorical
variable. We utilized multivariate binary logistic regression in the following statistical design. In each case univariate analysis was performed to determine the significance of each of the potential risk factors for the primary outcome, thereby identifying potential confounding variables to be included in the multivariate model. Variables found to be associated with the dependent variable (primary outcome) on univariate logistic regression at a probability threshold less than 0.20 were included in the multivariate logistic regression model. Donor Age and pre-transplant viral load were the only two variables found to be associated with the dependent variable. Statistical significance was defined as $p<0.05$ and all tests were two-tailed (where applicable). Statistical analysis was performed using the SPSS software program (version 15.0 for Windows, SPSS, Inc., Chicago, IL).

**V.1.6 RESULTS**

Forty-eight patients met the study criteria. Among those patients, study samples were not obtained from liver biopsies in four; four patients had early recurrence and needed treatment prior to enrolment, and three patients refused to participate. Thus we report on 37 HCV-LT. Among these patients, 17 had measurements performed at six-months and were prospectively followed up to their
12 months liver biopsy while the other 20 were assessed only at 12 months.

For patients at 12 months post transplant, 16 had recurrence and 21 had no recurrence based on the METAVIR Score. Demographic data for the two groups are presented in Table 1. No differences were found regarding age, gender distribution, pre-transplant viral load, ALT, genotype; type of immunosuppression, or donor age between the two groups. As well no differences were noted regarding anthropometric data: BMI, BIA, or WHR (Table 1). However, regarding liver histology, there was a significant difference between the two groups regarding liver inflammation. Forty one percent of HCV-LT with recurrence had ‘moderate’ inflammation, whereas only 5% of patients with no recurrence had ‘moderate’ inflammation (p=0.022) (Table 2).

HCV-LT with recurrence at 12 months had higher hepatic LPO levels (µmol MDA/gram of liver tissue) compared to those with no recurrence (1.66 ± 0.270 versus 0.878 ± 0.128, p=0.015) (Table 3). No differences were seen in liver or plasma AOP between the two groups (Table 3). No differences were found between the two groups regarding plasma antioxidant levels (Table 4) or macronutrient intake (Table 5) or micronutrient antioxidant intake (Table 6).
A significant relationship was seen between liver LPO and HCV disease recurrence and this significance continued when accounting for pre-transplant viral load and donor age. Due to our small sample size and most relevant factors identified in the literature as risk factors involved in the progression of disease recurrence, we conducted analyses on only two variables: pre-transplant viral load and donor age.

HCV-LT who were followed at both six and 12 months post transplant had similar hepatic inflammatory activity and hepatic steatosis at both time frames (Table 7). However, HCV-LT with recurrence at 12 months had significantly higher hepatic LPO (µmol MDA/gram of liver tissue) at six-months when compared to HCV-LT with no recurrence (1.86 ± .619 versus 0.746 ± 0.143, p=0.038).
### V.1.6. Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Recurrence (n=16)</th>
<th>No recurrence (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>55.4 ± 1.3</td>
<td>54.7 ± 1.3</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>% of Patients</td>
<td>% of Patients</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>76</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27.3 ± .81</td>
<td>28.3 ± .87</td>
</tr>
<tr>
<td><strong>BIA (% Fat)</strong></td>
<td>24.4 ± 2.2</td>
<td>22.1 ± 2.3</td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>.959 ± .01</td>
<td>.927 ± .02</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td>% Patients</td>
<td>% Patients</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>58</td>
</tr>
<tr>
<td><strong>Serum ALT (U/L)</strong></td>
<td>69.1 ± 10.1</td>
<td>61.8 ± 9.4</td>
</tr>
<tr>
<td><strong>Genotype % 1,1A, 1B</strong></td>
<td>% Patients</td>
<td>% Patients</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>70</td>
</tr>
<tr>
<td><strong>CsA</strong></td>
<td>% Patients</td>
<td>% Patients</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>70</td>
</tr>
<tr>
<td><strong>Donor Age &gt;45 y</strong></td>
<td>% Patients</td>
<td>% Patients</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td><strong>Pre-Tx Viral Load (IU/ML)</strong></td>
<td>2.1 x 10⁵ ± 1.2 x 10⁵</td>
<td>3.7 x 10⁶ ± 3.2 x 10⁶</td>
</tr>
</tbody>
</table>

**Abbreviations:**  
*BMI:* Body Mass Index;  
*WHR:* waist-to-hip ratio;  
*ALT:* serum alanine aminotransferase;  
*CsA:* cyclosporine A  
Data expressed as Mean ± SEM or percent patients  
Patients with recurrence: 19% Genotype 3; no recurrence: 24% Genotype 3 (p=.404).  
No significant differences between the two groups.
### V.1.6. Table 2. Liver Histology

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>No recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM (n=16)</td>
<td>Mean ± SEM (n=21)</td>
</tr>
<tr>
<td><strong>Fibrosis Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hepatic Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Minimal-Mild</td>
<td>53</td>
<td>76</td>
</tr>
<tr>
<td>Moderate</td>
<td>41*</td>
<td>5*</td>
</tr>
<tr>
<td><strong>Hepatic Steatosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Fat</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>&lt;33%</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>33-66%</td>
<td>11</td>
<td>9</td>
</tr>
</tbody>
</table>

*Chi-square comparing HCV-LT with recurrence and moderate hepatic inflammation with HCV-LT no recurrence, \( p=0.022 \). No other significant differences.
### V.1.6. Table 3. OxS Measurements

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>No recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mean ± SEM</strong></td>
<td><strong>Mean ± SEM</strong></td>
</tr>
<tr>
<td>(n=16)</td>
<td>(n=21)</td>
<td></td>
</tr>
<tr>
<td><strong>Liver LPO</strong></td>
<td>1.66 ± .279*</td>
<td>.878 ± .128*</td>
</tr>
<tr>
<td>(µmol MDA/g liver tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Liver AOP</strong></td>
<td>24.4 ± 5.7</td>
<td>20.6 ± 3.2</td>
</tr>
<tr>
<td>(µmol uric acid/g liver tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma AOP</strong></td>
<td>.09 ± .02</td>
<td>.05 ± .01</td>
</tr>
<tr>
<td>(µmol UAE)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** LPO: liver peroxidation; MDA: malondialdehyde; AOP: Antioxidant Potential; UAE: uric acid equivalents. Data were log transformed and independent sample t-test was conducted; statistical significance, p<.05

* Liver LPO with recurrence compared to no recurrence p=0.015
## V.1.6.Table 4. Plasma Antioxidant Levels

<table>
<thead>
<tr>
<th></th>
<th>Recurrence (n=16)</th>
<th>No recurrence (n=21)</th>
<th>Ref Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>♦ Retinol (µmol/L)</strong></td>
<td>2.64 ± .468</td>
<td>3.72 ± .483</td>
<td>1.5- 3.04</td>
</tr>
<tr>
<td><strong>Gamma-tocopherol (µmol/L)</strong></td>
<td>2.97 ± .629</td>
<td>2.67 ± .403</td>
<td>2-7</td>
</tr>
<tr>
<td><strong>Alpha-tocopherol (µmol/L)</strong></td>
<td>21.8 ± 4.14</td>
<td>20.9 ± 1.5</td>
<td>&gt;12</td>
</tr>
<tr>
<td><strong>Vitamin C (µmol/L)</strong></td>
<td>66.8 ± 12.3</td>
<td>54.5 ± 5.7</td>
<td>23-84</td>
</tr>
</tbody>
</table>

♦ all trans retinol. No significant differences were found between the two groups regarding plasma micronutrient antioxidant levels.
### V.1.6. Table 5. Macronutrient Intake

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>No Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SEM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td>(n=21)</td>
</tr>
<tr>
<td><strong>Total Calories</strong></td>
<td>1671 ± 138</td>
<td>1757 ± 165</td>
</tr>
<tr>
<td><strong>Total Protein</strong></td>
<td>72 ± 5.4</td>
<td>82 ± 12</td>
</tr>
<tr>
<td><strong>Protein, % of energy</strong></td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total Carbohydrate</strong></td>
<td>197 ± 15</td>
<td>212 ± 20</td>
</tr>
<tr>
<td><strong>CHO, % of energy</strong></td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td><strong>Total Fat</strong></td>
<td>68.7 ± 10.9</td>
<td>65.2 ± 6.4</td>
</tr>
<tr>
<td><strong>Fat, % of energy</strong></td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td><strong>PUFA</strong></td>
<td>11.0 ± 1.9</td>
<td>10.1 ± 1.7</td>
</tr>
<tr>
<td><strong>Total Fiber</strong></td>
<td>18.6 ± 2.3</td>
<td>15.5 ± 1.7</td>
</tr>
</tbody>
</table>

**Abbreviations:** *PUFA*: Polyunsaturated fatty acid. No significant differences were seen in macronutrient intakes between the two groups. Patients were meeting DRI for most nutrients with the exception of fiber where 100% of patients failed to meet the Estimated Average Requirement (EAR).
### V.1.6. Table 6. Micronutrient Intake

<table>
<thead>
<tr>
<th></th>
<th>Recurrence Mean ± SEM (n=16)</th>
<th>No recurrence Mean ± SEM (n=21)</th>
<th>Ref range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (µg/d)</td>
<td>951 ± 246</td>
<td>889 ± 172</td>
<td>625(µg/d)</td>
</tr>
<tr>
<td>% NOT MEETING EAR</td>
<td>47</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg/d)</td>
<td>103.7 ± 18.5</td>
<td>84.1 ± 17.4</td>
<td>75(mg/d)</td>
</tr>
<tr>
<td>% NOT MEETING EAR</td>
<td>59</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg/d)</td>
<td>5.9 ± 1.2</td>
<td>5.1 ± 0.83</td>
<td>12(mg/d)</td>
</tr>
<tr>
<td>% NOT MEETING EAR</td>
<td>84</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** EAR: Estimated Average Requirements.
Reference ranges were based on the Dietary Reference Intakes (DRI), Food and Nutrition Board, Institute of Medicine, National Academies, 2002. Majority of patients were males thus DRIs based on males. No significant differences between the two groups regarding micronutrient antioxidant intake.
### Table 7. Liver Histology and Hepatic Lipid Peroxidation in a Subgroup of Patients at Six-Months Post-Transplant and Their Status at 12 Months Post-Transplant

<table>
<thead>
<tr>
<th></th>
<th>Recurrence at 12 mo (n=6)</th>
<th>No Recurrence at 12 mo (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver Histology</strong></td>
<td>% of patients</td>
<td>% of patients</td>
</tr>
<tr>
<td><strong>Inflammation-6 m</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>minimum-mild</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Inflammation-12 m</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>minimum-mild</td>
<td>67</td>
<td>82</td>
</tr>
<tr>
<td>moderate</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td><strong>Hepatic Steatosis-6 m</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>90</td>
<td>71</td>
</tr>
<tr>
<td>&lt;33%</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>33-66%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hepatic Steatosis-12 m</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>&lt;33%</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>33-66%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hepatic Lipid Peroxidation</strong> (µmol MDA/g tissue)</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>6 mo post-transplant</td>
<td>1.86 ± 0.62**</td>
<td>.75 ± 0.14**</td>
</tr>
</tbody>
</table>

Recurrence at 12 months indicates mild fibrosis

** p=0.038.

No other statistical significance between the two groups.
V.1.7 Discussion

The results of this study indicate that lipid peroxidation, determined by hepatic levels of malondialdehyde was significantly higher in HCV-LT with disease recurrence than in recipients with no recurrence.

This study is unique because it is the first comprehensive study to describe hepatic OxS, plasma antioxidants, nutritional intake and status in HCV-LT with and without disease recurrence, at 12 months post transplant. HCV-LT with recurrence were more oxidatively stressed when compared to those with no disease recurrence. In addition, HCV-LT with HCV disease recurrence at 12 months were more oxidatively stressed at six-months post transplant, a finding that preceded the histological changes supportive of disease recurrence.

A number of risk factors contributing to disease recurrence have been reported in the literature. These include host factors: pre-transplant viral load (360); type of immunosuppression (361); use of steroid bolus for acute rejection episodes (362) and donor age >45 years (355). When examining donor age less than 45 or greater than 45 years of age we found no difference in those with or without recurrence. A previous study, conducted at our center (206) reported donor age as the sole independent predictor of fibrosis progression over a 5-year time frame in multivariate analysis in a large sample size
(206). The possible reasons for these differing results may be attributed to the differing designs, the differing sample size and the differing endpoints used.

The presence of OxS post transplant and its possible role, as a risk factor in HCV disease recurrence has never been studied. HCV-LT have universal viral re-infection (363), infection leads to inflammation contributing to OxS. In HCV patients, OxS occurs due to both the viral core protein itself (328) and the host immunologically-mediated response (157). Not surprising, significantly higher levels of hepatic inflammation and significantly higher hepatic LPO were seen in HCV-LT with recurrence when compared to those with no recurrence. An interesting finding was that HCV-LT with recurrence at 12 months did not have different levels of hepatic inflammation at six-months, but did have significantly higher hepatic LPO. It is reasonable to speculate that HCV viral proteins may be responsible for the increased hepatic LPO reported in HCV-LT. This has been demonstrated in the transgenic mouse model where ROS overproduction occurred in the absence of inflammation (364) and in another study, the presence of MDA adducts were detected in areas of active fibrogenesis (171). This may support the hypothesis that hepatic LPO contributes to disease recurrence and that OxS is increased due to other factors.
OxS can be influenced by smoking (239), alcohol (365), diabetes (356) and obesity (366). Liver specific factors positively influencing OxS include inflammation, hepatic steatosis (332) and iron (169). There was no difference in the two groups regarding obesity, WHR, or diabetes. Both patient groups were overweight/obese and a significant number of patients had type 2 diabetes mellitus. Hepatic steatosis can lead to fibrosis and cirrhosis (332) however we report no difference in degree of hepatic steatosis between the two groups and of interest, we note that the majority of patients had ‘no fat’ on 12 month liver biopsy. Increased OxS can occur as a result of increased iron storage resulting in liver damage (169). However, iron stains were negative in all HCV-LT and thus it is unlikely that increased iron was a major cause of increased OxS.

Hepatic AOP levels were similar between the two groups. This could be partly explained by the fact that those with recurrence at 12 months had only mild fibrosis and quite likely had a sufficient antioxidant defense system. This is in agreement with Yadav and colleagues (174) who reported that the severity of inflammation did not affect liver levels of antioxidants, however increasing fibrosis was associated with decreased liver antioxidant levels indicating that severe disease may be a consequence of antioxidant depletion or decreased liver storage resulting from fibrosis (174). To the best of
our knowledge there have been no studies examining hepatic AOP or individual antioxidants in HCV-LT with and without disease recurrence.

Altered plasma levels of micronutrient intakes can lead to increased OxS however, we report no difference between the two patient groups and all patients had ‘within’ reference ranges for all micronutrients. In other HCV patient populations, findings from antioxidant vitamin supplementation studies have shown improvement in plasma antioxidant levels but supplementation had no affect on viral load (177, 179, 180). In direct contrast to this, two studies have reported that antioxidant supplementation improved both plasma levels and decreased viral load (181) with a 2-point reduction in histological activity index in 25% of the patients studied (182). No antioxidant studies have been conducted in HCV-LT in association with HCV disease recurrence.

Nutritional factors such as low antioxidant intakes and/or high intakes of PUFA can increase OxS. Diets high in PUFA have been shown to increase the susceptibility to OxS (301-303). High fat diets can lead to overproduction of oxidants positively influencing OxS and this has been demonstrated in other disease conditions such as diabetes (356) cystic fibrosis (82) and CVD (367). Our results indicated no difference between the two groups regarding PUFA intake. Furthermore, no differences were seen regarding micronutrient
antioxidant intake between the two groups to account for increased OxS. As previously reported, neither patient group met their DRI for Vitamin E.

Immunosuppressive medications have been shown to increase oxidant production resulting in OxS. Research has shown that Cyclosporine A (CsA) is more proinflammatory than TAC (368), however the majority of the patients were prescribed CsA, which is the current standard therapy for this patient population.

This study has some limitations. The cross-sectional design of one component of this project did not allow for determination of causation. As well, the data generated from the second component of the study (prospective design) will need to be interpreted with caution due to the small sample size. It could be argued that due to the small sample size and controlling for only 2 risk factors associated with HCV disease recurrence some crucial factors influencing outcomes could have been missed. To adequately conduct multivariate binary logistic regression 10-20 patients per variable would be needed. Unfortunately, our sample size consisted of approximately 40 patients and thus we were limited to analyzing two possible factors associated with accelerated disease progression.

**V.1.8 Conclusions**

In conclusion, on the basis of increased hepatic lipid peroxidation, HCV-LT are oxidatively stressed. In a small prospective study, this finding was observed even though HCV-LT with higher hepatic LPO levels had no significant differences in hepatic inflammation when compared to HCV-LT with no recurrence.
larger longitudinal study to confirm these findings and future studies to determine the effect of vitamin supplementation on OxS and HCV disease recurrence may further shed light on this process.
CHAPTER VI: GENERAL DISCUSSION

In this thesis, we investigated the possible association between OxS and post-transplant complications, with BOS in lung transplant and with HCV disease recurrence in liver recipients. Our results showed a higher level of OxS in patients with these complications. The question is whether OxS contributes to the pathogenesis of these complications or is a consequence or both.

In the lung disease population, increased oxidative stress has been adequately documented in pre-transplant (43, 82, 85, 245, 369-372) and as well, early post lung transplantation, in relation to Ischemia-Reperfusion injury (IRI). However, OxS in long-term lung recipients is not well studied (57) and there is minimal information regarding the role of OxS and the development of BOS. Similarly in HCV liver recipients, minimal research has been conducted examining OxS in this patient population.

The cross-sectional design used in our studies suggests an association between OxS and post-transplant complications, either with BOS for lung transplant or with HCV disease recurrence for patients with HCV who had a liver transplant. In addition, preliminary results from the cohort study in liver transplant patients suggest that OxS precedes these complications, at least for HCV disease recurrence. A cohort study for lung transplant patients was not possible due to
conflict with other on-going studies in this relatively small patient population.

The studies of this thesis are the first step in exploring the role of OxS in the pathogenesis of BOS and HCV disease recurrence and our results support a potential contribution with significant increase in lipid peroxidation parameters in patients with severe BOS compared with non-BOS patients and in HCV disease recurrence versus no disease recurrence. Furthermore, preliminary data from the cohort study in liver recipients suggest that OxS is present even before the development of the histological changes associated with disease recurrence thus lending support for a role in the pathogenesis of this complication. This is supported by studies in liver disease such as HCV and non-alcoholic fatty liver disease showing that lipid peroxidation in the liver, in addition to producing tissue damage, may contribute to disease progression by inducing more inflammation and fibrosis via increased production of cytokines and activation of stellate cells (373) (374).

The most important limitation of this series of studies was the observational nature of the design. Cross-sectional studies examine only associations but, on the other hand, these types of studies are seen as the first step to a cohort study or an eventual intervention trial. This design was appropriate in our patient population considering
the lack of data in the post-transplantation literature and the difficulties conducting prospective cohort or intervention studies in these patients because of the complexity of their medical care and the small population. This design was used to better define the demographic and clinical characteristics, particularly related to OxS and nutritional parameters, of the study groups and detect potential differences between groups and associations.

In BOS, we detected a higher level of lipid peroxidation, as determined by BALF LPO, in those who had the most severe form of BOS (BOS 2-3) when compared to those with mild BOS. However, plasma antioxidants and nutritional intakes were not different. It is possible that this increase in OxS was due to increase lung inflammation alone. We did not detect any difference in any other parameters known to influence OxS such as level of antioxidants or nutritional intakes or in other potential contributors such as infection, drugs or BMI. Due to the nature of the population, none of these patients were current smokers.

It was hypothesized that there might be a difference in antioxidants between those with and without disease considering that, in addition to causing tissue damage, lipid peroxidation increase the requirements of antioxidants to scavenge the free radicals and interrupt the cascade reaction. On the other hand, the sample size may have been too
small to detect a difference in plasma antioxidants or nutritional intake. For example, based on sample size calculation for nutritional intakes, at least 675 patients per group would have been necessary to detect a clinically important statistical difference in dietary antioxidant intake. However, this large sample size would have been impossible to reach considering that 100 lung transplants are conducted annually.

For future directions, a prospective cohort study may have helped determine if the presence of OxS was associated with poorer outcome such as the development of BOS or a more severe form of BOS. Finally, an intervention trial with antioxidant supplements may have helped support a causal relationship between OxS and BOS where variables such as FEV$_1$ and BALF would have been assessed at baseline and at 1 year as per transplant protocol. Unfortunately, this second study, cohort or intervention pilot study could not be performed due to conflict with other studies in an already small patient population. This is one of the reasons why we decided to pursue our studies in the liver recipients and assessed OxS in HCV disease recurrence.

In HCV liver recipients, we also used a cross-sectional design to first determine if HCV-LT had higher liver LPO compared to a Control group and also compared to HCV-Non-transplant patients. We were able to determine that not only did the HCV-LT have OxS, evidenced
by higher hepatic LPO levels at six-months post transplant, when compared to the Control group, but also in addition, these patients had higher liver LPO levels when compared to HCV non-transplant patients. Furthermore, higher hepatic LPO was seen in HCV-NT when compared to controls, lending support to the hypothesis that oxidative stress is implicated in HCV disease.

HCV-LT and HCV-NT both had significantly higher WHR when compared to controls. It is therefore possible that increased abdominal obesity (WHR) contributed to increased OxS. As well, HCV-LT had lower plasma AOP when compared to controls, but not when compared with non-transplant HCV patients. Gamma-tocopherol plasma levels were also higher in both HCV groups (LT and NT) compared to controls but not when compared to each other.

In HCV-LT, we again used a cross-sectional design to determine if there was a difference in OxS between HCV disease recurrence and no disease recurrence. Additionally, we were able to follow a subgroup of patients prospectively and determine that those who developed disease recurrence at 12 months not only had increased liver lipid peroxidation at the time of the diagnosis but, in a subgroup of these patients, there was also evidence of increase liver lipid peroxidation at six-months, therefore preceding the histological changes diagnostic of disease recurrence. These results provide stronger support that OxS
participates in the pathogenesis of HCV disease recurrence. In addition, we are the first to report that there is an increase in liver lipid peroxidation in HCV disease recurrence and that lipid peroxidation precedes disease recurrence. The latter needs to be confirmed in a larger cohort study (on-going). An intervention study, if feasible, looking at the effect of antioxidant vitamins on parameters such as OxS, HCV viral load or rate of disease recurrence would support these findings.

**Future Directions**

Intervention studies have been performed in the HCV non-transplant populations and several trials showed some beneficial effects on disease progression. RCTs examining patients with CHC have reported improvements in plasma ALT with (i) supplementation of 500 mg Vitamin E (177); (ii) 500 mg Vitamin E in combination with 750 mg Vitamin C as adjunctive therapy with antiviral medications (178) and (iii) phenol-rich supplement (180). Decreased viral load has also been reported in RCT in patients with CHC supplemented with NAC 1800 mg, plus Selenium 400 µg along with 500 mg Vitamin E. As well, a clinical trial in 50 CHC patients supplemented with an antioxidant mixture containing ascorbic acid, and alpha-tocopherol and several other antioxidants, reported decreased viral load. It would be of interest to conduct similar studies in HCV-LT.
All these studies are very challenging to conduct in the transplant populations. These patients frequently have co-morbidities, are on multiple drug regimens and have frequent setbacks such as rejection, infections and even malignancies. Many of these factors may affect OxS. Considering this clinical complexity and the fact that the transplant population is small compared to the populations of patients with other more common conditions, conducting an interventional study using antioxidant vitamins will present significant challenges. In addition, it will be difficult to avoid contamination as many patients are already taking multivitamins or have easy access to these supplements and therefore, a placebo controlled arm may be very difficult to do.

It is also important to keep in mind that, in the broader context, research studies examining antioxidant supplementation in patients with a variety of disease conditions such as Atherosclerosis (375) and Alzheimer’s disease (376) have been disappointing, underlying the difficulties regarding decisions for dosing, timing, type and combination of supplements, duration of the intervention and outcome measures. Results from primary and secondary intervention trials with 400 IU vitamin E every other day plus 500 mg vitamin C daily have reported increased mortality in both HC and patients with diseases (377-379). The evidence is even more convincing that antioxidant
supplementation with the goal of reducing the risk of Cardiovascular Disease is not supported by the findings from current RCT (380,381). Similarly, 400 IU vitamin E every other day along with daily 500 mg vitamin C supplementation, neither alone nor together, reduced the risk of prostate or total cancers (382-384). However, some of these studies have been criticized for not examining an oxidative stress biomarker, thus making it difficult to identify individuals who may have benefited from supplementation (385). Therefore, based on these past studies, optimal antioxidant vitamin supplementation studies are challenging to design.

Another potential intervention would be a dietary modification (386). Initiating nutrition strategies to improve the total antioxidant capacity (TAC) of lung and liver recipients’ diets represents a possible dietary alteration. Some authors agree that improving the patients overall nutrition intake may prove more beneficial than antioxidant supplementation (387). As well, some authors have reported that high TAC foods (consisting of red berries, spinach, coffee, olive oil among others) can minimize inflammation (388-392). However, these types of diets can be more difficult to follow compared to a recommendation of consuming an additional pill, but may have other beneficial compounds that have not yet been identified (393).
Our results provide support for future research on the role of OxS and antioxidants in the post transplant population particularly in HCV liver recipients. Before recommending antioxidant supplementation in this population, we should assess its benefit and determine what is the appropriate dose and combination, as well as the duration of treatment (394,395). We may also want to determine if we can select out the ‘more oxidized’ transplant patient at an earlier stage for supplementation with extra antioxidants (396). Furthermore, we will need to examine if supplementation leads to minimization of the inflammatory process evident in chronic rejection and HCV disease progression? If so, would this reduce OxS, and as a result, have a clinical impact on FEV$_1$ and the development of BOS, or would it reduce inflammation and minimize the progression of HCV disease recurrence?

The main challenge for the next few years, through continued research endeavors, will be to answer our question: **Does OxS CONTRIBUTE TO THESE COMPLICATIONS OR IS IT A CONSEQUENCE OR BOTH?** Currently there is insufficient evidence to answer this question.

**In conclusion,** this thesis has documented a higher level of OxS in BALF of lung transplant patients with severe BOS and in the liver of liver recipients with HCV disease recurrence. In general, this was not
associated with significant differences in antioxidant micronutrients either in plasma or in the diet.

These findings add to the body of knowledge regarding OxS in post-transplant recipients and suggest that in these patients, antioxidant supplementation should be further studied, to assess its effect on OxS and clinical outcomes such as FEV$_1$ for BOS and histology or HCV viral load for HCV disease recurrence.
Chapter VII: References


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APPENDICES

Appendix 1. Ascorbic Acid procedure

Appendix 2. Additional chemicals for Retinol, Vitamin E, Beta-Carotene Protocol

Appendix 3. Consent form Lung Transplant Recipients

Appendix 4. Consent form HCV liver recipients

Appendix 5: The METAVIR Staging System

Appendix 6: Curriculum Vitae
APPENDIX 1. ASCORBIC ACID PROCEDURE

Reagents:

Meta-phosphoric acid: mixture of 35% meta-phosphoric acid (HPO₃) with 65% stabilizer as sodium metaphosphate (NaPO₃). Store at room temperature. Skin irritant. For this assay, treat the material in bottle as 100% MPA and ignore the 35-65% ratio.

Sulfuric acid concentration. Store at room temperature. Very irritant.

2.4-dinitrophenylhydrazine, FW 198.1, store at room temperature. Inflammable, toxic and carcinogenic.

Thiourea, FW 76.12, store at room temperature. Toxic and carcinogenic.

Cupric sulfate pentahydrate, FW 249.7, store at room temperature. Toxic.

Preparation of solutions:

5% meta-phosphoric acid (5% MPA): dissolve 25 g of MPA in 400 ml of deionized water and dilute to final volume of 500 ml. Store at 4°C for up to 1 week.

10% meta-phosphoric acid (10% MPA): dissolve 10 g of MPA in 80 ml of deionized water and dilute to final volume of 100 ml. Store at 4°C for up to 1 week. If left at room temperature, MPA will undergo molecular rearrangement and loss of assay activity. MPA left at room temperature for longer than 2-3 hrs should not be used for assay.

4.5 mol/L sulfuric acid (25% sulfuric acid). With caution, carefully add 250 ml of concentrated sulfuric acid (18 mol/L) to 750 ml of deionized water and mix well. Store at 4°C.

65% sulfuric acid. With caution, add 1300 ml concentration sulfuric acid (18 mol/L) to 700 ml of ice-cold deionized water and mix well. Store at 4°C.

2.2% 2,3-DNPH. Dissolve 1.21 g of 2,4-DNPH in 55 ml of 4.5 mol/L sulfuric acid. Mix well and then filter until solution is clear. Store at 4°C for up to 2 weeks.

5% Thiourea. Dissolve 5 g of thiourea in 80 ml of deionized water. Mix well, and dilute to 100 ml with deionized water. Store at 4°C for up to 2-4 weeks.
1% copper sulfate. Dissolve 0.78 g of cupric sulfate -5 hydrate in 40 ml of deionized water. Mix well, and dilute to 100 ml with deionized water. Store at 4°C for up to 2-4 weeks.

Color reagent. Combine 2,4-DNPH, thiourea and cupric sulfate solutions (20:1:1). During preparation, add thiourea to 2.4-DNPH and mix well prior to adding cupric sulfate. Store at 4°C and prepare only enough solution to be used within 4 hours.

**Preparation of standards for standard curve:**

Stock L-ascorbic acid standard. Weight out 10-20 mg – ascorbic acid powder and prepare a 1 mg/ml solution using 5% MPA. Prepare and use daily, store at 4°C until discarded.

Add 400 µl of stock std. To 9600 µl of 5% MPA. 4 mg/dl
Add 300 µl of stock std to 9700 µl of 5% MPA 3 mg/dl
Add 200 µl of stock std to 9800 µl of 5% MPA 2 mg/dl
Add 100 µl of stock std to 9900 µl of 5% MPA 1 mg/dl
Add 4 ml of 1.00 mg/dl std to 4.0 ml 5% MPA 0.5 mg/dl
Add 2 ml of 0.5 mg/dl std to 2.0 ml 5% MPA 0.25 mg/dl
Add 5ml of 5% MPA only 0 mg/dl

Vitamin C concentrations in serum/plasma are determined using calculated slope and intercept based on the absorbance readings of L-ascorbic acid standards at 521.0 nm. Seven tests of three standards are used for these calculations. Vitamin C concentrations are derived based on the equation: \( x = \text{absorbance reading at 521 nm} - \frac{\text{absorbance intercept}}{\text{slope}} \).

**This method determines total biological active vitamin C** concentrations (the sum of L-ascorbic acid + dehydro-L-ascorbic acid in biological material) spectrophotometrically using 2,4-dinitrophenylhydrazine as chromogen (Bessey, O.A., et al, J. Biol. Chem. 168:197:1947) as modified for use in the NHANES 2- Health and Nutrition Survey.

**Preparation of samples**
Obtain serum/plasma sample (preferred specimens is either serum or EDTA plasma). After sample collection, sera (plasma) is obtained by centrifugation (1800 x g, 10 min; 4°C) and an equal volume of serum/plasma is added to an equal volume of 10% meta-phosphoric acid. Samples are mixed by vortex and stored at −85°C. All samples are analyzed in duplicate and the values reported as the average of two separate determinations.

Note: If only a limited sample volume is available, prepare acidified sample extract using one smaller part sample plus on matched part 10% MPA (ex. 200 µl sample to 200 µl of 10% MPA).
APPENDIX 2. ADDITIONAL CHEMICALS FOR RETINOL, VITAMIN E, BETA-CAROTENE PROTOCOL

Chemicals Needed

HPLC-grade n-hexane (Sigma)
HPLC-grade ethanol (Sigma)
HPLC-grade Methanol (Sigma)
Beta-hydroxy toluene (BHT)
Stabilized ether (Sigma)

Supplies needed

Autosampler Vials: Varian Part number: 392611549
Clear vials, red caps, ptfe/silicone liners

Inserts: Varian part number: 392611595
200 ul inserts, silanred glass, conical point, polymer spring

Column: Varian, 150 x 4.6 mm, Res Elut 5u C18 90 A, cat number: 1215-9012

Standards

All standards have to be stored either in -20C or -70 C. Follow the instruction on the bottles

dl-rac- alpha-tocopherol acetate (Sigma)
RRR-gamma-tocopherol (Sigma)
Beta-carotene (Sigma)
Retinol (Sigma)
Lycopene (Sigma)

Solutions

- Prepare 35 mg of BHT in 100 ml of ethanol

- Mobile phase: 95% HPLC-Grade Methanol for the first 2.5 minutes and pure 100% HPLC-grade methanol for the remaining of the run.

-Prepare an ether-methanol solution by mixing 90 ml of HPLC grade methanol and 30 ml of stabilizing ether.

Preparation of Standard Solutions
**Beta-Carotene Stock (100 mg/L):** dissolve 10 mg of β-carotene in 5 ml of dichloromethane and dilute to 100 ml with ethanol/BHT. This stock solution can be stored at -70 C for 1 month

**Standard dilution (500 µg/L):** Dilute 1.0 ml of the stock solution and make it to 20 ml with ethanol-BHT. Immediately before use, dilute 1:10 in ethanol-BHT by taking 1 ml of previous dilution and making it to 10 ml with ethanol-BHT

**Retinol Stock solution (120 mg/dL):** dissolve 0.0120 g of (all E) retinol in 10 ml of ethanol-BHT. This stock solution can be stored at -70 C for approximately 1 month.

**Alpha-Tocopherol Stock Solution (372 mg/dL):** dissolve 0.0372g of alpha-tocopherol in 10 ml of ethanol/BHT. This stock solution can be stored at -70 C for approximately 2 months.

**Gamma-Tocopherol Stock Solution (60 mg/dL):** dissolve 0.0060g of gamma-tocopherol in 10 ml of ethanol/BHT. This stock solution can be stored at -70 C for approximately 2 months.

**Standard Stock Solution:** Prepare a mixture of stock solutions using: 41.6µL Retinol, 250 µL Gamma-Tocopherol, 242 µL Alpha-Tocopherol and dilute to 5 mL.

**Standard 2:** Prepare a mixture of 200 µL of stock solution and 200 µL of ethanol/BHT. Prepare Daily.

**Standard 3:** Prepare a mixture of 100 µL of standard and 100 µL of ethanol/BHT. Prepare Daily.

**HPLC Conditions**
- Column temperature: 37 C
- Flow rate: 1.7 ml/minute
- Pressure: approximately 50 bar
- Injection volume: 100 µl
- Run time: 50 min
- Multi-wavelength detector: program at 3 different wavelengths (292, 325, and 450 nm for tocopherols, retinol, and carotenoids respectively)
Appendix 3. Consent form Lung Transplant Recipients

INFORMED CONSENT
AND PATIENT INFORMATION

STUDY TITLE: An examination of the nutritional factors affecting the Severity of Bronchiolitis Obliterans Syndrome (BOS), in the Lung Transplant Population. A Pilot Study. Version date: Sept 06

INVESTIGATORS: Dr. Lianne Singer, Dr. Johane Allard and Janet Madill

Patient Name ___________________________ Date ______________________

You are being invited to participate in this research study.

The researcher will explain the purpose of the study. He or she will explain how the study will be carried out and what you will be asked to do. The researcher will also explain the possible risks and possible benefits of being in the study. You should ask the researcher any questions you have about any of these things before you decide whether you wish to take part in the study. This process is called informed consent.

This form also explains the research study. Please read the form and talk to the researcher about any questions you may have. Then, if you decide to be in the study, please sign and date this form in front of the person who explained the study to you. You will be given a copy of this form to keep.

Nature and Purpose of the Study

You have been asked to participate in this study as either a patient with BOS (with chronic rejection), or as a patient without BOS, (no chronic rejection). We will look at your levels of anti-oxidants (vitamin and minerals) in your blood and BALF. Patients post lung transplant have lowered 5-year survival rates due to chronic rejection, the actual cause is not known; studies have suggested that some patients may be oxidatively stressed in which case some of the vitamin and mineral that you eat or some of the vitamin and minerals in your lungs are low. These low levels or intakes may be related to BOS.

The sponsor of this project is the Ontario Respiratory Care Association.

EXPLANATION OF PROCEDURES
Two ml of fluid (BALF) (less than 1 tablespoonful) that has been previously collected from your routine bronchoscopy and is currently stored in the freezer will be removed to analyze antioxidants and OxS markers. There are no additional costs to you.

**Discomforts and Risks**

There are no discomforts or risks involved.

**Benefits**

You will not benefit directly from participating in this study, but the information gathered may contribute to the knowledge of the nutritional factors underlying BOS.

**Alternative Therapies**

If you decide not to participate, or withdraw after entering the study, the post transplant care you would normally receive will be provided.

**Confidentiality**

Any information that is obtained about you during the course of this study will be kept strictly confidential. Any research data obtained about you in this study will not identify you by name, only by your initials and coded number. Your name will not appear in any reports published as a result of this study. However, your medical records and charts and test with your name on them will be made available to the investigator, for viewing purposes. This is for the purpose of verifying information obtained during this study.

While the results of the research study will probably be shared with other people and may be published in scientific reports, your name and the fact that you were in the study will be kept confidential.

**Refusal/Withdrawal**

The decision whether to be in this study is entirely up to you. Participation is voluntary. Also, if you decide now to participate, you will be able to change your mind later and withdraw from the study.

There will be no penalty or loss of health care benefits if you decide not to participate, or if you withdraw from the study.
Compensation

If you become ill or are physically injured as a result of participation in the study, medical treatment will be provided. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.

Rights and Complaints

If you have any questions about your rights as a participation in a research study, you may contacts the following neutral individual: Dr Heslegrave, Chair of the Research Ethics Board at the University Health Network at 416 340 4557

If you wish to discuss future questions or concern you may have about this study, or if you need help with any health problem, illness or injury, you may contact the study doctor Dr Allard at 416 340 5159; or the Dietitian Janet Madill at 416 340 4800 Ext. 2738 or Dr. Lianne Singer, Medical Director of the Lung Transplant Program, at 416 340 4800 Ext 4996.

I ACKNOWLEDGE THAT I HAVE READ THE ABOVE EXPLANATION OF THIS STUDY THAT ALL OF MY QUESTIONS HAVE BEEN SATISFACTORILY ANSWERED, AND I AGREE TO PARTICIPATE IN THIS RESEARCH STUDY.

I have been given a signed copy of this consent

__________________________________________________________ Date

Signature of patient

I CERTIFY THAT I HAVE EXPLAINED FULLY TO THE ABOVE PATIENT THE NATURE AND PURPOSE, PROCEDURES AND THE POSSIBLE RISK AND POTENTIAL BENEFITS OF THIS RESEARCH STUDY.

__________________________________________________________ Date

Signature of researcher or designate
Appendix 4. Consent form HCV liver recipients

STUDY TITLE: Oxs and Nutritional Factors in HCV post liver transplant patients.

INVESTIGATORS: DR. Johane Allard, Dr. Les Lilly, Dr. George Therapondos, Dr. Gary Levy and Janet Madill PhD(C) RD

You are being invited to participate in this research study.

The researcher will explain the purpose of the study. He or she will explain how the study will be carried out and what you will be asked to do. The researcher will also explain the possible risks and possible benefits of being in the study. You should ask the researcher any questions you have about any of these things before you decide whether you wish to take part in the study. This process is called informed consent.

This form also explains the research study. Please read the form and talk to the researcher about any questions you may have. Then, if you decide to be in the study, please sign and date this form in front of the person who explained the study to you. You will be given a copy of this form to keep.

Nature and Purpose of the Study

You have been asked to participate in this study as a patient with Hepatitis C Virus (HCV). Fifty to 60% of HCV patients with disease recurrence require some type of treatment by 1-year post transplant. As well, patients with HCV disease recurrence have lowered 5-year survival rates post transplantation, but the actual cause is not known. Studies have suggested that some patients may be oxidatively stressed in which case some of the vitamins and minerals that you eat or some of the vitamins and minerals in your blood or liver are low. These low levels or intakes may be related to development of HCV disease recurrence.

We will look at your levels of antioxidants (vitamin and minerals in your blood) and in your liver tissue at 6 and 12 months post transplant. These time frames will correspond with your regularly scheduled blood work and biopsies.

EXPLANATION OF PROCEDURES

Your standard care during your post-transplant clinic visit will remain unchanged except for additional 5 ml of blood (~ 1 tablespoonful), that will be taken at 6 and 12 months post transplant. This sample will be drawn during your regularly scheduled blood work so no extra needle pricks are necessary. As well, a very small amount of tissue from your regularly scheduled liver biopsy will be taken at 6 and 12 months post transplant for analysis. The blood and liver tissue will be tested for levels of vitamins.
and minerals. All other information needed will be obtained from your routine visit medical history forms (such as: results of liver biopsy, all other blood work information). Your blood sample and any remaining liver tissue will be destroyed at the end of this study.

There are no additional costs to you.

**DISCOMFORTS AND RISKS**

Risks associated with drawing blood from your arm include pain, bruising, lightheadedness and, on rare occasions, infection.

**Benefits**

You will not benefit directly from participating in this study, but the information gathered may contribute to the knowledge of the nutritional factors underlying HCV recurrence.

**Alternative Therapies**

If you decide not to participate, or withdraw after entering the study, the post transplant care you would normally receive will be provided.

**Confidentiality**

If you agree to participate in this study, the study doctor and his/her study team will look at your personal health information in order to collect the information that is needed for the study. Personal health information is any information about you, such as your name, address, date of birth, new or existing medical records, or the types, dates and results of medical tests or procedures, that could be used to identify you. Only information that is needed for the study will be collected from your personal health information. The information that is collected for the study will be kept in a locked and secure area by the study doctor for a period of 7 years. These records will only be available to the study team or to those people or groups listed below. Your participation in this study also may be recorded in your medical record at this hospital.

Representatives of the University Health Network Research Ethics Board may look at the study records and at your personal health information to check that the information that was collected is correct and to make sure that the study was done according to applicable laws and guidelines.

All information collected during this study, including your personal health information, will be kept confidential and will not be released to anyone outside the study unless required by law. You will not be identified in any reports, publications, or presentations that may come from this study.
If you decide to leave the study, the information about you that was collected before you left the study will still be used. No further information will be collected without your permission except to follow up on safety events that might have occurred during your participation in the study.

Participation

Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without affecting your medical care.

Compensation

If you become ill or are physically injured as a result of participation in the study, medical treatment will be provided. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.

Rights and Complaints

If you have any questions about your rights as a participant in a research study, you may contacts the following neutral individual: Dr Heslegreave, Chair of the Research Ethics Board at the University Health Network at 416 340 4557

If you wish to discuss future questions or concern you may have about this study, or if you need help with any health problem, illness or injury, you may contact the study doctor Dr. Johane Allard at (416) 340 5159, or the Dietitian Janet Madill at (416) 340-4800 Ext. 2738

I ACKNOWLEDGE THAT I HAVE READ THE ABOVE EXPLANATION OF THIS STUDY THAT ALL OF MY QUESTIONS HAVE BEEN SATISFACTORILY ANSWERED, AND I AGREE TO PARTICIPATE IN THIS RESEARCH STUDY.

I have been given a signed copy of this consent.

Printed Name:_________________________________________________

Signature of participant                                     Date
I CERTIFY THAT I HAVE EXPLAINED FULLY TO THE ABOVE PATIENT THE NATURE AND PURPOSE, PROCEDURES AND THE POSSIBLE RISK AND POTENTIAL BENEFITS OF THIS RESEARCH STUDY.

___________________________________________________________
Person obtaining consent (print name)                      Date

___________________________________________________________
Signature of person obtaining consent                     Date
Appendix 5: The METAVIR Staging System (284)

F0 No fibrosis

F1 Stellate enlargement of portal tracts but without septum formation

F2 Enlargement of portal tracts with rare septum formation

F3 Numerous septa without cirrhosis

F4 Cirrhosis
Appendix 6: Curriculum Vitae

Over 23 years successful experience as a leader in evidence based research as a clinical dietitian, striving towards professional achievement as a Clinician-Scientist. I have inspired others to think outside a traditional mindset, in an attempt to merge the scientific worlds of Nutrition and Medicine, through the promotion of research. My commitment to the dietetic profession can be seen through my various contributions toward improving the quality of life and health for transplant patients.

Professional Education and Development

2003  University of Toronto, Institute of Medical Science, PhD program
1999  University of Toronto, Department of Nutritional Sciences
      Master of Science
1989  International Board of Certified Lactation Consultant Examination
1982  American Dietetic Association Registration Examination
1982  Dietetic Internship  Vancouver General Hospital
1981  University of Manitoba, Winnipeg, Manitoba
      Bachelor of Human Ecology (Nutrition Major)

Career Profile

2006-present  Research Advisor/Transplant Dietitian

2000-2006  Research Practice Leader/Transplant Dietitian
            University Health Network, Toronto General Hospital, Toronto

In addition to my transplant responsibilities, effectively provide educational resource and ongoing coaching, counseling and mentoring, to support 44 dietitians and dietetic interns to facilitate evidence based research.

1990-2000  Senior Dietitian for Transplantation

            University Health Network, Toronto General Hospital, Toronto
Successfully assessed, planned and implemented nutrition interventions for transplant patients by staying abreast of current research. Proactively educated and investigated alternative therapies for transplant patients.

1984-1990

**Clinical Dietitian for General Medicine & Surgery**

**University Health Network**, Toronto General Hospital, Toronto

Successfully developed and implemented the existing protocol for Nutrition assessments within the transplant program. Through this, championed new relations with physicians and dietitians.

**Research Grants**

2009


2008


2007


Schembri N, Stillman S, Brum, M, Madill, J, Holloway T and Figliano M. The impact of increased intervention with a Registered Dietitian and Exercise Physiologist on overall health outcomes of patients attending Cardiac Rehabilitation at Toronto Western Hospital. University Health Network, Allied Health Granting Agency, UHN, Toronto, ON.

Fixler R, Scott L, MacGarvie D, Madill J, Blunt V and Miller C. Do enterally fed patients in the medical surgical intensive care units at UHN achieve their recommended nutritional intakes? University Health Network, Allied Health Granting Agency, UHN, Toronto, ON.

Carroll L, Joshi N, Bell H, Madill J, Nash C and Campbell F. Is body mass index an appropriate tool for assessing nutritional status in Asian, maintenance hemodialysis patients as compared to subjective global assessment? University Health Network, Allied Health Granting Agency, UHN, Toronto, ON.

2006


Gismondi L, Murphy A, Madill J and Camelon K. Does nutrition intervention for patients with type 2 diabetes or prediabetes lead to changes in dietary intake and activity level? University Health Network, Allied Health Granting Agency, UHN, Toronto, ON.

2005


Figliano M, Dickinson H, Lavelle T, Camelon K, Madill J and Hoffman, L. Do patients recovering from anorexia nervosa have a better chance of staying well if they expand their range of food? Allied Health Research Funds, UHN, Toronto, ON.


2004

Madill J. An examination of the nutritional factors affecting the severity of Bronchiolitis Obliterans Syndrome (BOS), in the lung transplant population. Ontario Respiratory Care Society.


2003

Madill J. A double-blinded placebo controlled trial of vitamin $\mathrm{B}_{12}$, $\mathrm{B}_{6}$ and folate supplementation for the treatment of Hyperhomocysteinemia in the lung transplant population. International Society for Heart and Lung Transplantation (ISHLT).

2002


2001

MacGarvie, D and Madill J. Development of standard and objective definitions for dysphagia diet fluid puree food viscosities. University Health Network, Allied Health Granting Agency, UHN, Toronto, ON.

Madill J. Body Mass Index as a predictor of mortality in the liver transplant population. Canadian Liver Foundation, Toronto, ON.
Madill J and Burden T. *An evaluation of a research initiative at UHN: “does it increase level of research activities of Dietitians?”* Novartis Inc., Toronto, ON.

Madill J. *Can nutrition intervention decrease lipid levels in drug-induced dyslipidemia?* Ontario Thoracic Society, Toronto, ON.

2000

Madill J. Dietitian Coordinator, Multi-site Trial. Fujisawa 004 study. *A randomized controlled clinical trial for the treatment of dyslipidemia in the liver transplant population.* Fujisawa Inc., Toronto, ON.

1999

Madill J. *Hyperhomocysteinemia in the liver transplant population.* Fujisawa Inc., Toronto, ON.

1997


Madill J. *Body Mass Index as a predictor of mortality in the lung transplant population.* Ontario Thoracic Society, Toronto, ON.

**TEACHING AWARDS**

2009  
*The Wightman-Berris Academy & University of Toronto Award.*  
For individual Teaching Excellence, nominated by Research colleagues, Ryerson volunteer students and dietetic interns.

2003  
*The Wightman-Berris Academy & University of Toronto Award.*  
For Individual Teaching Excellence, nominated by Ryerson volunteer students, dietetic interns and colleagues.

2000  
*The Wightman-Berris Academy & University of Toronto Award.*  
For Individual Teaching Excellence, nominated by Ryerson volunteers students, dietetic interns and colleagues.

**SPECIAL AWARDS**

2008  

**OTHER AWARDS**
2004  UHN, Nursing and Allied Health Scholarship Award for PhD Research Endeavors

2002  Keynote speaker: Interns graduation ceremony, University Health Network, Toronto, ON.

1993  Clintec Nutrition Practice Based Research Award
      Canadian Dietetic Association Achievement Awards

1991  Ross Clinical Dietetics Award: For outstanding achievement in the profession of dietetics.

**Facilitation of Learning in Practice**

1990 to current  On a yearly basis, have successfully coordinated and facilitated learning activities and have successfully mentored 30 volunteer nutrition students from University of Toronto and Ryerson University. A significant number of these students have gone on to complete a dietetic internship and then to become successful dietitians. Many of these students have provided positive feedback that I was an inspirational role model and an outstanding teacher, and that this played a large part in their own successful careers.

1986 to current  On a yearly basis, have coordinated and facilitated dietetic internship placements, both as a coordinating and resource dietitian.
Publications

2009


Madill J. Invited to write Practice Based Evidence in Nutrition (PEN) knowledge pathways for liver and lung transplant patients. This is a new initiative set out by Dietitians of Canada (will be published in Dec 09).

2008


2006

Madill, J. Invited to contribute a chapter on Nutritional Status of Lung Transplant Recipients, in Lung Transplantation, edited by Dr. Chaparro and Dr. Keshavjee (published 2007).

2005


2003


Invited to contribute a chapter Nutrition Support in textbook entitled Principles and Practice of Liver Transplantation. Edited by Dr. Gary Levy and Dr. David Grant, to be published in May 2003.

2002

2001


1996


1994


1993


1990


1989


1984


1982
**Madill J.** The role of the dietitian in a consumer setting. Canadian Consumer, October 1982.

**CV: PRESENTATIONS**

2009


**Madill, J.** Arendt, B; Aghdassi, E; Chow, C-W; Guindi, M; Therapondos, G; Lilly, L and Allard JP. Oxidative Stress and Nutritional Factors in HCV Liver Recipients. Poster presentation at CST/CAT annual conference. March 2009. Banff, AB, Canada.

2008


**Madill, J.** Oxidative Stress and Nutritional Factors in Lung Recipients. Poster session. ASPEN International Conference, Feb 2008, Chicago, IL, USA.

**Madill, J.** Invited Speaker to conduct research workshop: Ready for Research! International Better Breathing Symposium, Feb 2008, Toronto, ON, Canada.


2007

Carroll L, Bell H, **Madill J** and Campbell F. Is body mass index an appropriate tool for assessing nutritional status in Asian, maintenance hemodialysis patients as compared to subjective global assessment? Poster presentation at St. Mikes Research Day, Sept 07, Toronto, ON.

Filxer R, Scott L, McGarvie D, Blunt V, **Madill J** and Miller C. Do enterally fed patients in the Medical/surgical intensive care units at UHN achieve their recommended intakes? Oral presentation at St. Mikes Research Day, Toronto, ON.
Schembri N, Stillman S, Brum M, Madill J, Holloway, Tanya and Figliano M. The Impact of Increased Intervention with a Registered Dietitian and Exercise Physiologist on Overall Health Outcomes of Patients Attending Cardiac Rehabilitation at Toronto Western Hospital. Poster Presentation at St. Mikes Research Day, Sept 07, Toronto, ON.

Madill J. Oxidative Stress and Nutritional Factors in BOS. Presented at the Better Breathing Symposium, Feb 07, Toronto, ON.

2006


Gismondi L, Murphy A, Camelon K and Madill J. Does nutrition intervention for patients with type 2 diabetes or prediabetes lead to changes in dietary intake and activity level. Presented at St. Mikes Research Day, Sept 06, Toronto, ON.

2005


Figliano M, Dickinson H, Lavelle T, Camelon K, Madill J and Hoffman L. Do patients recovering from anorexia nervosa have a better chance of staying well if they expand their range of food? Presented at St. Mikes Research Day, Sept 05, Toronto, ON.

Fanasabian T, Noel M, Bell H, Dowdy K, Madill J and Mori M. What is the prevalence of over-and underweight patients in the UHN hemodialysis population? Presented at St. Mikes Research Day, Sept 05, Toronto, ON.

2004


Madill J, Allard J, Singer L. An examination of the nutritional factors affecting the Severity of Bronchiolitis Obliterans Syndrome (BOS), in the Lung Transplant Population. Presented at Institute of Medical Science, University of Toronto, Jan 2004, Toronto, ON.
Thompson, C and Madill J. Is the use of self-administered vitamin, mineral, multivitamin and herbal supplements pre-transplant associated with increased acute rejection episodes within 90-days post surgery? Presented at Allied Health Research Day, Feb 04, Toronto, ON.

2003


Madill, J. Is the use of self-administered vitamin, mineral, multivitamin and herbal supplements pre-transplant associated with increased acute rejection episodes within 90-days post surgery? Presented at Day in Transplants, Toronto, ON, Nov 03.

2002


Noseworthy M, Mori M, and Madill J. Relationship between nutrition intervention and pre-dialysis BUN levels: A pilot study. Presented at UHN dietitian’s seminar, July 02 and St. Mikes Research Day, Sept 02, Toronto, ON.


Minicucci S, Mori M and Madill J. The self-administered use of vitamin, mineral, and complementary supplements in the lung transplant population at the University Health Network. Presented at UHN dietitian’s seminar, July 02 and St. Mikes Research Day, Sept 02, Toronto, ON.

Madill J, Mollot K, Gutierrez C and Keshavjee S. Nutrition intervention is effective in treating drug-induced dyslipidemia in Lung Recipients. Presented at CAT, May 02, Mont Tremblant, QC.

Madill J, Mollot K, Gutierrez C, Keshavjee S. Dietitians can make a difference: Examining dyslipidemia in the Lung Transplant Population. Presented at Dietitians of Canada, June 01, Winnipeg, MA.

2000


1999
Madill J. Beyond your borders: Using results of clinical nutrition research as important selection criteria in liver transplant patients. Presented at CFDR/DC conference, 1999, Vancouver, BC.


1998


1997


1996

Madill J, Mori M, Gutierrez C and Kesten S. The effect on serum lipid levels of lung transplant patients after changing from CSA to Tacrolimus (FK506). Presented at Royal College, CAT/CTS, Sept 1996, Halifax, NS.


1995


1994


1993

Madill J, Wong C, Levy G and Greig P.D. Diabetes following liver transplantation. Results of the University of Toronto Liver Transplant Program. Published in ODA


1992


1990


*underlined indicates presenters