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WEB-BASED STUDIES OF LIFESTYLE FACTORS AND IMMUNE FUNCTION

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**Karolinska
Institutet**

Stockholm 2011

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ISBN 978-91-7457-177-6

Printed by



www.reproprint.se

Gårdsvägen 4, 169 70 Solna

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Abstract

Upper respiratory tract infection (URTI), is estimated to cost \$40 billion per year in the US, not including the cost of influenza, and is the most common reason for seeking primary care in many countries. Despite this, little is known about how to decrease susceptibility. Lifestyle factors such as physical activity, stress, sleep, and diet are important modulators of immune function and in this thesis, five papers evaluating the use of Internet for data collection, and the association of lifestyle factors to URTI and immune function in blood, are described.

In **Paper I**, the feasibility of using Web questionnaires compared with traditional paper questionnaires in a population-based setting was investigated. The use of interactive Web-based questionnaires resulted in lower initial response rate but similar total response-rates on follow-up questionnaires. Based on these findings, we conducted a population-based Web cohort study of 1509 Swedish men and women aged 20-60 with a follow-up period of four months (**Papers II-IV**). Participants reported a total of 1181 occurrences of URTI. In **Paper II**, results show that high levels of physical activity (≥ 55 MET-hours/d, MET, metabolic equivalent task) were associated with an 18% reduced risk of self-reporting URTI compared with low levels of physical activity (< 45 MET-hours/d) (IRR 0.82, 95% CI 0.69-0.98). In addition, highly stressed people, particularly men, appeared to benefit more from physical activity than those reporting lower stress levels. When studying intake of antioxidants and URTI risk in **Paper III**, we found that a high intake of vitamin C from food (> 200 mg/d) was associated with a 31% lower risk of URTI compared with a low intake (< 100 mg/d) (IRR 0.69, 95% CI 0.49-0.98) among women. This association was not seen among men, who overall had a lower intake of vitamin C than women. In **Paper IV**, we assessed adherence to the Nordic Nutrition Recommendations (NNR) as a measure of an overall healthy diet. The NNR include recommendations on macronutrients (e.g. saturated fat), micronutrients (vitamins and minerals), fiber, sodium, alcohol, and physical activity. Good adherence versus poor adherence to the NNR was not associated with risk of URTI in this study. In **Paper V** the natural variations of sleep duration, stress, physical activity, leukocyte numbers, and their function was examined in 36 men and women, aged 20-54. Results show that short sleep duration (< 7 h) prior to blood draw was associated with a 49% higher T cell function (95% CI 7/109%) in response to phytohemagglutinin (PHA) and 30% lower natural killer cell activity (NKCA) (95% CI -46/-8%) compared with normal prior sleep (7-9h). Results also indicate that high general perceived stress was associated with a 39% higher T cell function in response to PHA (95% CI 0/94%), and that high general physical activity was associated with an increased number of B cells and T cells, but general physical activity was not clearly associated with immune cell function.

In conclusion, we found that Web questionnaires can be useful for research purposes in populations with high proportion of Internet users, especially when multiple follow-ups are needed. **Paper II** and **III** conclude that lifestyle factors such as physical activity, stress, and diet are associated with risk of self-reported URTI. However, no association was seen between good adherence to the NNR and URTI, which may be explained by the fact that the participants were generally very well-nourished and with a limited variation in the NNR score (**Paper IV**). In **Paper V**, we found that lifestyle factors were related to immunological markers in blood. Natural short sleep duration was associated with lower NKCA, which might impair ability to fight infections.

1 LIST OF PUBLICATIONS

- I. Bälter KA, Bälter O, Fondell E, Lagerros YT
Web-based and mailed questionnaires: a comparison of response rates and compliance
Epidemiology 2005;16(4):577-9.
- II. Fondell E, Lagerros YT, Sundberg CJ, Lekander M, Bälter O, Rothman KJ, Bälter K
Physical activity, stress and self-reported upper respiratory tract infection
Medicine & Science in Sports & Exercise Epub June 23 2010
- III. Fondell E, Bälter O, Rothman KJ, Bälter K
Dietary intake and supplement use of vitamins C and E and upper respiratory tract infection
Submitted
- IV. Fondell E, Christensen SE, Bälter O, Bälter K
Adherence to the Nordic Nutrition Recommendations as a measure of a healthy diet and upper respiratory tract infection
Public Health Nutrition Epub September 21 2010
- V. Fondell E, Axelsson J, Franck K, Ploner A, Lekander M, Bälter K, Gaines H
Short natural sleep is associated with higher T cell and lower NK cell activities
Submitted

Related publications:

Fondell E, Bälter O, Bälter K, Lagerros YT

How Can a Person Without Computer Experience Participate in a Web Survey?

Poster presentation, CHI 2003, Fort Lauderdale, Florida, USA. April 5-10 2003.

Fondell E, Johansson ALV, Lagerros YT, Sundberg CJ, Lekander M, Bälter O,

Rothman KJ, Bälter K

Physical Activity and Susceptibility to Upper Respiratory Tract Infection

Oral presentation, ISEI 2007, Sendai, Japan

Fondell E, Johansson ALV, Lagerros YT, Sundberg CJ, Lekander M, Bälter O,

Rothman KJ, Bälter K

The Effect of Physical Activity and Chronic Stress on Self-Reported Upper Respiratory Tract Infection

Poster presentation, PNIRS 2008, Madison, Wisconsin, USA

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2 LIST OF ABBREVIATIONS

BMI	Body mass index
BMR	Basal metabolic rate
CI	Confidence interval
CM	Culture medium
d	Day
DG	Degrees of freedom
DGA	Dietary guidelines for Americans
FASCI	Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood
FANKIA	Flow-cytometric Assay of Natural Killer cell Immune response in Activated whole blood
FFQ	Food frequency questionnaire
GEE	Generalized estimating equations
h	Hour / hours
IRR	Incidence rate ratio
MET	Metabolic equivalent task
NK cell	Natural Killer cell
NKCA	Natural Killer cell activity
NNR	Nordic Nutrition Recommendations
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PSS	Perceived stress scale
PUFA	Polyunsaturated fatty acids
PWM	Pokeweed mitogen
SD	Standard deviation
SIIDC	Swedish Institute for Infectious Disease Control
SEA+B	Staphylococcus enterotoxin A/B
URTI	Upper respiratory tract infection

*“I love the doctors, they are dears,
but must they spend such years and years
investigating such a lot
of illnesses which no one's got,
when everybody, young and old,
is frantic with the common cold?
And I will eat my only hat
if they know anything of that.”*

– Sir Alan Patrick Herbert (1890-1971)

3 INTRODUCTION

Our immune system has long been viewed as an autonomic system within our body. In recent years, however, it has become recognized that the immune system is involved with other systems in the body, including the endocrine system, the nervous system (1, 2), the muscular system (3), and is affected by nutrient intake (4). The immune system is a very complicated defense system and many of its functions and how it is regulated remain unknown today.

A major task for the immune system is to combat infectious agents. One of the most common infections, and the most common reason for seeking primary care in many countries is upper respiratory tract infection (URTI), which includes the common cold and influenza (5). Annual costs of URTI, including cost of doctors visits and loss of work days, is estimated to be about \$40 billion in the US (6), not including the cost of influenza. Despite this, little is known about strategies to reduce susceptibility.

Lifestyle factors such as physical activity, stress, diet, and sleep are potential modulators of immune functions and may also influence URTI risk. Moderate physical activity has been associated with reduced inflammation and has been suggested to boost immune responses to pathogens (7, 8). Regular physical activity may also result in a higher number of circulating NK cells, which could increase the ability to fight infections (9). Stress, if long-term, is thought to downregulate all immune responses (10). Dietary intake is also related to immune function. For example, dietary antioxidants neutralize free radicals in the body and reduce oxidative damage, which may play a role in inflammatory diseases (11), heart disease (12), cancer (13), neurological diseases (14), and URTI (15). In addition, short and disturbed sleep has been shown to increase the risk of URTI (16), but there is still uncertainty on the exact mechanism responsible (17, 18).

Most previous studies on lifestyle factors and immune function have focused on special groups or situations, for example studies on physical activity have focused on athletes, studies on diet have focused on supplement use, and studies on sleep have focused on induced severe sleep deprivation. In this thesis, a more general approach to study lifestyle factors is used. This thesis explores physical activity and perceived stress in everyday life, dietary patterns and intake of vitamin C from food and supplement use and their relation to URTI. In addition, natural variations of sleep, physical activity, and stress are explored in relation to markers of immune function in blood.

Studies on URTI require frequent follow-ups to capture URTI incidence. The feasibility of using Web questionnaires for frequent follow-ups is also explored in this thesis.

4 BACKGROUND

This chapter will give a brief introduction to immunology, URTI, and a brief introduction to possible lifestyle factors that may be associated with immune function.

4.1 IMMUNE FUNCTION

Immunology as a science is relatively new. The origin of modern immunology is sometimes attributed to Edward Jenner's vaccinations with cowpox to prevent smallpox in Massachusetts in 1796 (1). The immune system is a complex system and its purpose is to protect the body from invading pathogens like virus, bacteria, fungi, or parasites. The skin and mucosal membranes are the first line of defense, being physical barriers to keep pathogens out. Should a pathogen enter the body, it will meet defenses from the innate immune system (innate, the part of immune system that we are born with) and most threats that the body encounters are eliminated quickly without causing any symptoms. Natural Killer (NK) cells are an important part of innate immunity, which purpose is to either eliminate or keep a viral infection under control while the adaptive immune system (adaptive, part of the immune system that is acquired and dependent on memory) with cytotoxic T cells and antibody producing B cells prepare to eliminate the infection.

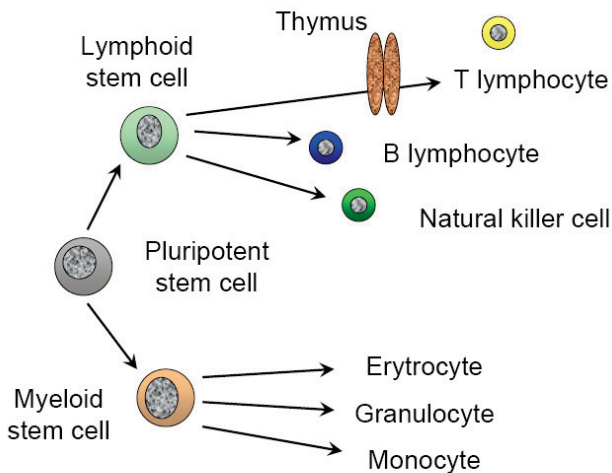


Figure 1. Development of immune cells.

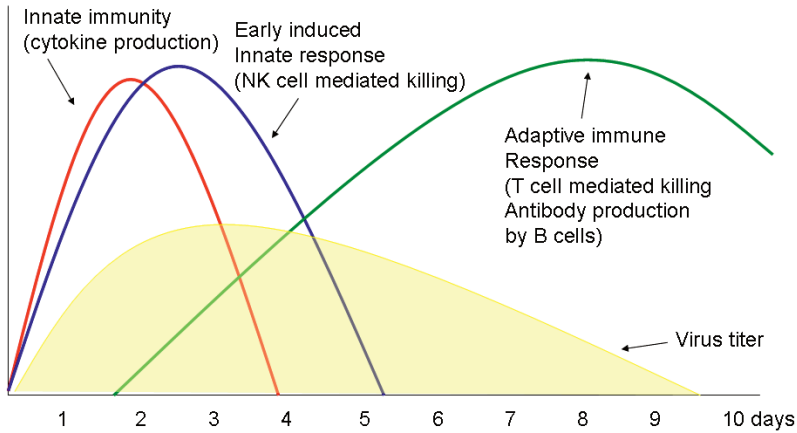


Figure 2. Timeline of the immune responses to a viral infection.

In addition to combating viral and bacterial infections, the immune system is also involved in combating cancerous tumor cells. The immune system is also a key player in inflammation, which is involved in cardiovascular disease, diabetes, autoimmunity, and allergies.

4.2 UPPER RESPIRATORY TRACT INFECTION

Adults, on average, get two to four URTI per year and most URTI are caused by a virus (19). Rhinovirus is the most common, causing 30-50% of all colds. Common cold can in rare cases be caused by fungus and bacteria (19, 20).

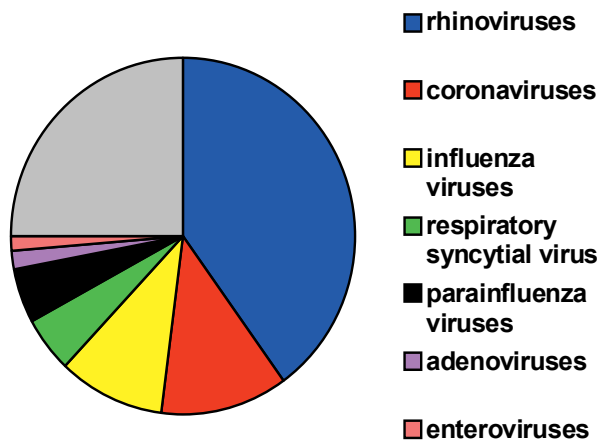


Figure 3. Viral causes of URTI (19, 21).

Most URTI in Sweden and countries with temperate climate occur during the colder winter season, presumably because in cold weather, people spend more time indoors, increasing the risk of transmission. The humidity is also lower during the winter

causing the inner lining of the nose to get dry and more vulnerable. In addition, viruses also more stable in cold, dry climates and survive longer outdoors. Viruses are transmitted through touching an infected area and then touching the eyes, nose or mouth, or by inhaling droplets that contain the virus in the air. The most effective way to prevent URTI is total isolation, but less extreme preventive methods include washing of hands, cough etiquette, virus-killing disinfectants, and influenza vaccine.

Symptoms of a common cold include nasal discharge, cough, sore throat, sneezing, and headache (22). Symptoms of influenza also include fever and muscle aches. Symptoms last 2 – 14 days, but most people recover within a week (22). Common colds can sometimes lead to bacterial infections, such as middle ear infections or sinus infections, and influenza can lead to pneumonia. There is no known cure for URTI, but there are ways to relieve symptoms through medications.

Because most URTI are of short duration, frequent follow-ups are needed for an accurate incidence tally. Therefore, for this research, we explored the use of Web-based questionnaires to assess incidence of URTI.

4.3 E-EPIDEMIOLOGY

In the field of epidemiology, large population studies are often used to study association between exposure (e.g. diet) and outcome (e.g. cancer). Until recently, most of these questionnaires have been printed and distributed by paper mail. The questionnaires in epidemiological research often contain hundreds of questions and can take hours to complete, and a majority of the printed questionnaires are returned incomplete, or with unreasonable answers to some of the questions. Participants are then often contacted by phone in order to complete missing answers or to correct unreasonable answers. Finally, the answers from the printed questionnaires need to be transferred to an electronic format. This manual handling is costly and offers a significant opportunity for cost reduction.

e-Epidemiology is defined as “the science underlying the acquisition, maintenance and application of epidemiological studies that are increasingly conducted through distributed global collaborations enabled by the Internet” (23). The Internet is a new tool for collecting self-reported information in epidemiologic studies. Web-based questionnaires are easy to administer and offer several advantages to paper questionnaires including immediate checks for incomplete or implausible answers, reminder messages to the respondent when a question is left unanswered, automatic summarization of answers, personalized feedback based on answers given, inclusion of illustrations or sounds to clarify complex questions, and hiding non-relevant follow-up questions. In addition, Web questionnaires require no expense for printing, postage, manual check of incomplete answers, and transfer of data to an electronic format. The major cost of the Web questionnaire is the system for handling the Web questionnaires and the design of the questions; thus, once the system is established, the extra cost to add a few thousand or even a few hundred thousand participants to the study is relatively small.

Internet access at home among men and women aged 16-74 in Sweden in 2004 was estimated to be 79%, and 76% used the Internet at least once a week (24). Internet access was slightly lower in certain groups, for example among the elderly, among women, among those with a lower education level, and among those born outside of

Sweden (24). Unfamiliarity with Web questionnaires or worries about security issues might also limit participation rates for some groups. However, as Internet access continues to increase, these differences are likely to diminish over time. In 2010, Internet access in Sweden was estimated to be 92.5% (25).

4.4 LIFESTYLE FACTORS

Our immune system is involved with other systems in the body. Factors associated with susceptibility to infections include nutrient intake, physical activity, stress, sleep, age, sex, body mass index (BMI), and viral exposure (e.g. hand washing, contact with small children, or use of public transportation). Below, the main exposures of interest in this thesis, physical activity, stress, dietary intake, and sleep will be described in detail, and other related exposures will be described briefly.

Table 1. Observational and intervention studies of moderate physical activity and URTI.

Reference, year	Type of study	Study time	Participants	Country	URTI measure (method and frequency)	Results
Schouten, W.J. et al. 1988 (26)	Descriptive (retrospective)	6 months	92 men, 107 women, age 20's	Holland	Self-reported retrospectively in questionnaire	Lower risk of URTI among women who exercised, but not among men
Nieman D.C. et al. 1990 (8)	Intervention	15 weeks	36 mildly obese women, aged 50-75 years	USA	Self-reported daily using log books	Lower risk of URTI among women in walking intervention group
Matthews, C.E. et al. 2002 (27)	Observational	12 months	547 men and women, aged 20-70 years	USA	Interview by clinician every 3 months	Lower risk of URTI among participants with higher levels of physical activity
Hemilä, H. et al. 2003 (28)	Observational	3 years	14401 men, aged 50-69 years	Finland	Interview by clinician every 4 months	No large association between common cold and level of physical activity
Chubak, J. 2006 (29)	Intervention	12 months	115 overweight and obese, sedentary postmenopausal women, aged 50-70 years	USA	Self-reported every 3 months in questionnaires	Lower risk of common cold among women in exercise intervention group, but no lower risk of influenza
Kostka, T. 2008 (30)	Observational	12 months	142 men, aged 33-90 years	Poland	Self-reported daily using log books	Lower risk of URTI among men with higher physical activity

4.4.1 Physical Activity

Several lines of research indicate that physical activity is beneficial for a person's health. Moderate physical activity has been associated with reduced risks of a large number of diseases, such as type 2 diabetes (31), cardiovascular diseases (32), certain cancers (33), and on overall mortality (34). Moderate physical activity has also been associated with reduced inflammation and has been suggested to boost immune responses to pathogens (7, 8).

Most studies on physical activity and immune function are limited to effects of acute exercise, where an increase in T cells, but not B cells can be observed during exercise, followed by a decrease in numbers after exercise (35). In addition, some studies suggest that Natural killer cell activity (NKCA) may be slightly elevated among rested athletes compared to sedentary controls (9, 36, 37).

A J-shaped association between physical activity and URTI was suggested in 1994 by David Nieman (38), in which moderate levels of physical activity were associated with a reduced risk of URTI, whereas very high levels of physical activity, such as for marathon-runners and elite cyclists, increased susceptibility to URTI. The majority of previous studies on physical activity and URTI have focused on athletes, and many have been conducted in small study populations, with fewer than 40 individuals (see Nieman (39) for a review). However, there are a few large studies on moderate physical activity and URTI. A cohort study of 20-70 year old men and women (N=547) found that moderate to vigorous physical activity was associated with a 20% lower risk of self-reported URTI (27). Another cohort study of 50-69 year old male smokers (N=14 400) reported a weak association near a null effect (RR 0.93, 95% CI 0.85-1.02) between physical activity and URTI (28). Consequently, little is known about the effects of moderate levels of physical activity on URTI.

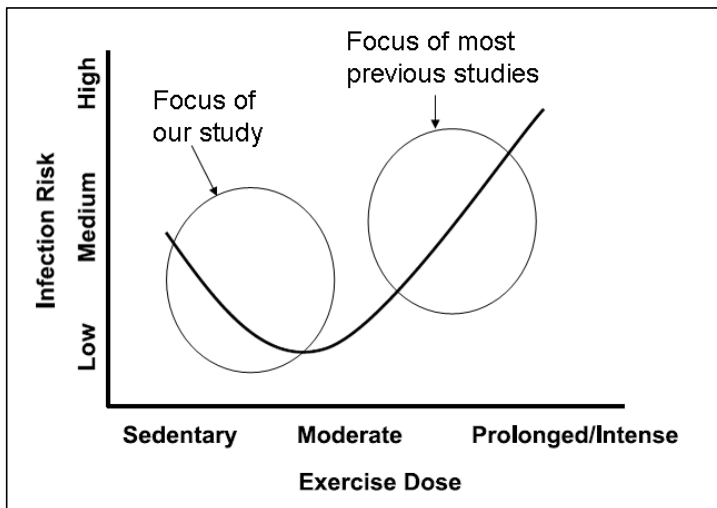


Figure 4. Illustration of the suggested J-shaped association between URTI and physical activity.

4.4.2 Stress

Stress is generally described with a circular definition, as *circumstances most people would find stressful* (10). There are two main different types of stress, acute stress that lasts hours or minutes (e.g. public speaking, examination), and long-term, chronic stress that lasts months or years (e.g. caring for a spouse with dementia) (40). Perceived stress is used to assess stress in this thesis (41). Perceived stress is a subjective method of measuring stress, is usually measured during the previous month, and is sometimes described as chronic stress.

Previous studies have found that acute stress upregulates innate immunity, and downregulates adaptive immunity, while chronic stress is associated with suppression of both cellular and humoral measures of immune function (10). Stress has been associated with numerous adverse health effects including cancer development (42-44), and increased markers for inflammation (45), which in turn are independent risk factors for cardiovascular disease (46). In addition, self-reported perceived stress during the previous month is associated with an increased risk of URTI (47, 48) (see Cohen (49) for a review).

Physically active subjects show attenuated cardiovascular and stress hormonal responses to mental stress as compared with physically inactive subjects (50). Given the corroboration from experimental animal models (51), it has been hypothesized that physical activity might protect against the immunosuppressive effects of chronic stress (52).

There may also be an association between stress and sleep. A bidirectional association has been reported where short sleep duration is associated with higher levels of cortisol and may exaggerate the effects of stress, while stress in turn can also cause poor sleep (53). The association between sleep and stress is also illustrated in a recent study where women with multiple awakenings mobilize fewer NK cells in response to a stressful task (54).

There are also proposed differences in physiological and behavioral responses to stress among men and women (55). The “fight-or-flight” response to stress, while present in both men and women, is proposed to be stronger in men, and a “tend-and-befriend” response is proposed to be more common in women in response to stress (55).

4.4.3 Diet

Ever since the early 1800’s, nutrients have been recognized for their regulatory effects on immune function (56). Most nutrients are needed for maintaining healthy immune functions and deficiency in almost any nutrient will lead to impaired immune functions (4).

Nutritional epidemiology is the study of dietary intake in epidemiology. Several important findings are attributable to nutritional epidemiology. One of the first examples is the discovery of citrus fruit curing scurvy in sailors by Lind in 1753 (57). The curative agent in citrus fruit, ascorbic acid (vitamin C), was not discovered until almost 200 years later when in 1937, Perla and Marmorston suggested that vitamin C

affects resistance to infections (58). Since the 1970s, vitamin C supplements have been sold for both preventive and therapeutic use for the common cold (58).

Dietary antioxidants neutralize free radicals in the body and reduce oxidative damage. These agents have been hypothesized to have a role in the occurrence of many diseases, including inflammatory diseases (11), ischaemic diseases (12), cancer (13), neurological diseases (14), and URTI (for a review, see (15)). The antioxidants vitamin C and vitamin E are the most studied antioxidants, and are the focus of Paper III. Other dietary components of special interest for immune function include vitamin A (59), vitamin D (60), zinc (61), selenium (62), and omega 3 fatty acids (4, 63-65).

4.4.3.1 Vitamins C and E

Vitamin C can be found in high concentrations in citrus fruits, bell peppers, Brussels sprouts, cabbage, cauliflower, broccoli, and berries. Vitamin E is mainly found in kiwi, nuts, egg, milk, avocado, spinach, and vegetable oils.

A recent review of over 30 trials studying the effects of vitamin C supplementation on common cold shows that vitamin C supplementation is associated with a modest 8% reduction in the duration of the common cold duration (95% CI 3% to 13%). There is also reportedly a reduction in incidence, but only among physically active individuals, for whom the risk ratio was 0.50 (95% CI 0.38, 0.66) (66).

Vitamin E is essential to protect the lipids in membranes from peroxidation. It has been shown to be associated with immune function and resistance to infections in both animal (67) and human (68) studies. Some randomized trials with elderly participants (see (69) for a review) showed a protective effect of vitamin E supplementation against the common cold, with a RR of 0.80 (95% CI 0.64-0.98).

Most previous studies on antioxidants and URTI have studied the effect of large doses through supplement use (66, 69, 70). Few studies have focused on antioxidants from food (71). One reason might be that the intake levels corresponding to supplement doses (~1g vitamin C /day or ~300 mg vitamin E/day) (72, 73) are hard to attain through a normal diet. Given that the dose, bioavailability, and exposure kinetics of antioxidants from food intake may differ from that of supplements, both dietary sources of these agents merit evaluation.

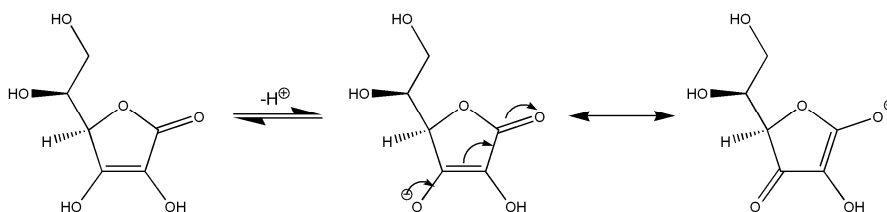


Figure 5. The antioxidative properties of ascorbic acid (vitamin C).

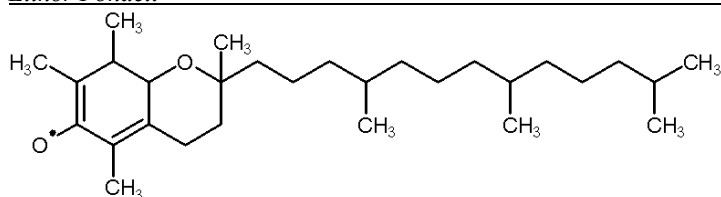


Figure 6. Structure of vitamin E (alpha-tocopherol).

4.4.3.2 Nutritional recommendations

The first nutrition recommendations date back to Britain in 1862. Following economic depression, the British Privy Council wanted guidelines for food that was cheap but would avoid diseases related to malnutrition (74). Today, most industrialized countries have nutrition recommendations and the Nordic countries have published joint recommendations; the *Nordic Nutrition Recommendations* (NNR) (75), since 1980. The NNR focus on the energy distribution from macronutrients, intake of micronutrients, and the most recent version from 2004 include physical activity. The NNR are based on the current nutritional situation in the Nordic countries and available scientific knowledge to promote overall good health and reduce the risk of diet-associated diseases.

A large number of studies have focused on effects of dietary patterns on chronic diseases, such as cardiovascular disease and cancer (76-80), for example, a lower risk of cardiovascular disease was found among women adhering to the Dietary Guidelines for Americans (DGA) (80). Less is known about the effects of dietary patterns on respiratory infections.

4.4.4 Sleep

Short sleep duration is becoming increasingly common in the industrialized world (81-83). Short and disturbed sleep has been shown to affect health in a number of ways, for example, increasing the risk of type 2 diabetes (84), URTI (16), cardiovascular risk factors (85, 86), breast cancer (87), and all-cause mortality (88). Some of these health problems are believed to be partially related to the effects of disturbed sleep on immune functions (17), but there is still uncertainty of the mechanism involved (17, 18).

Previous studies have found altered distribution of NK cells and reduced NKCA in response to induced partial sleep loss (89-91). Other effects of partial sleep deprivation include higher T cell proliferative responses to phytohemagglutinin (PHA) (92), as well as lower B cell activity (measured by immunoglobulin release) (92) (see (17) for a review on sleep and the immune system). In addition, pro-inflammatory mediators (IL-1-beta and TNF (Tumor Necrosis Factor)-alpha) that often increase in response to disturbed sleep (93) are directly involved in sleep regulation (94). This indicates an overlap between immune and sleep physiology that make short-term co-variations between the systems reasonable.

Most previous studies on sleep and immune function have focused on induced severe sleep deprivation (often including substantial manipulation with no sleep for 24-48 hours). Few studies have addressed natural variations of sleep duration, and these have failed to confirm the findings of severe sleep deprivation (95). It has been suggested, however, that severe sleep deprivation may affect immune functions differently than

mild sleep deprivation (17), indicating a need for more studies on mild sleep deprivation, natural sleep, and immune function.

4.4.5 Potential Confounding Factors

In order to study the associations of lifestyle factors and URTI, it is important to measure potential confounding factors. Below, some of these potential confounding factors are discussed.

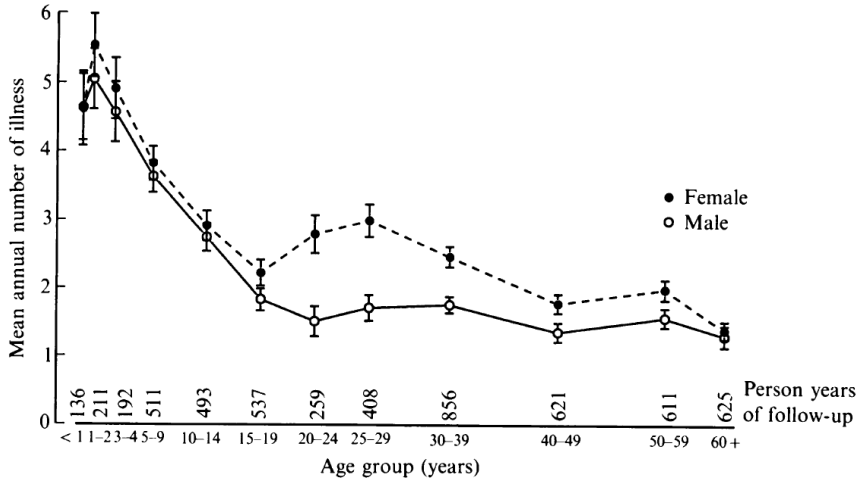


Figure 7. Mean number of respiratory illnesses by age and sex in a study by Monto et al. (20).

The incidence of URTI is highest during the earliest years of life and then decrease with increasing age up to around 65 after which URTI risk is increased due to immunosenescence (20, 96). Previous studies show that URTI frequency is higher among women in their 20's and 30's than among men in the same age groups (20). The Center for Disease Control and Prevention reported in 2009 that obese adults are at higher risk of morbidity and mortality from the H1N1 influenza (97). A recent study among school children also show that obese children are more likely to have bronchitis, and may be more likely to have a serious cold or influenza than children of normal weight (98).

When young children are sick in URTI, the viral concentrations in the nasal discharge may last longer than in adults. In addition, young children may also have poor cough etiquette and contact with young children is therefore important to measure when studying URTI. Additional potential confounding factors that might increase the risk of contracting a URTI is use of public transportation or regular contact with large crowds.

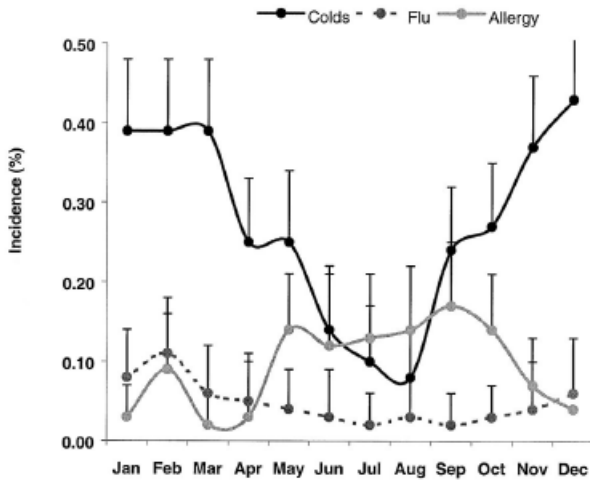


Figure 8. Seasonal variation in URTI and allergy. Massachusetts 1994-1998
 Matthews et al. (27).

The time of year is also important when studying URTI risk. A cold climate is known to increase the risk of URTI, possibly because dry air makes airways more vulnerable to infections. During the cold season, people are more likely to gather indoors than outdoors, which can increase the spread of infections. Other factors that might reduce the URTI risk are hand washing and influenza vaccination. Symptoms of pollen allergy are very similar to URTI symptoms and it can be difficult to distinguish the two. It is therefore crucial to gather information about pollen allergy if the study is during the pollen season. Information about certain chronic conditions (e.g. HIV) or medications (e.g. chemotherapy) that can weaken the immune system is also important to gather, as is information on smoking, which has been shown to aggravate and prolong URTI symptoms (99).

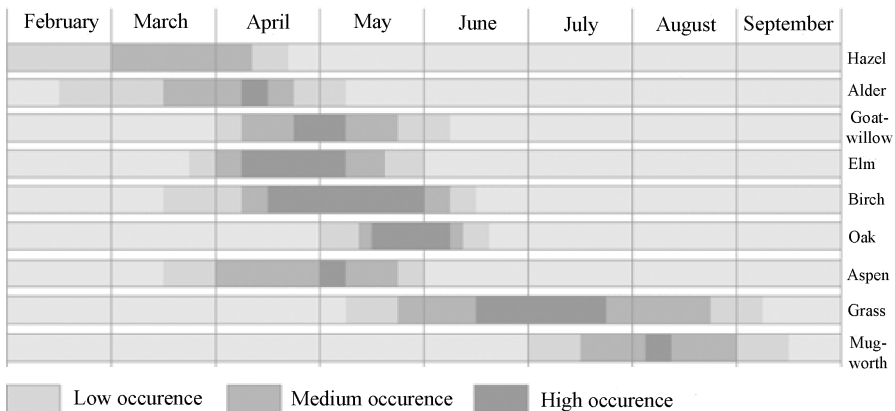


Figure 9. The pollen season in central Sweden in 2004. Add a 1-2 week delay for pollen in Northern Sweden where the LIME study was conducted. Data from the Palynological laboratory, Swedish Museum of Natural History, Stockholm.

5 AIMS

The overall aims of this thesis were to develop and evaluate the use of Web-based questionnaires, to study the association of lifestyle factors and self-reported URTI, and to study the association of lifestyle factors and immunological markers in blood.

The specific aims were to:

- develop, use and evaluate Web-based questionnaires in a population-based epidemiological study (Papers I-IV)
- study the association of physical activity, perceived stress and URTI (Paper II)
- study the association of vitamins C and E from diet and supplement use and URTI (Paper III)
- assess adherence to the NNR and study the association between adherence to the NNR and URTI (Paper IV)
- study the association of physical activity, perceived stress, and natural sleep and T cell-, NK cell-, and B cell function (Paper V)

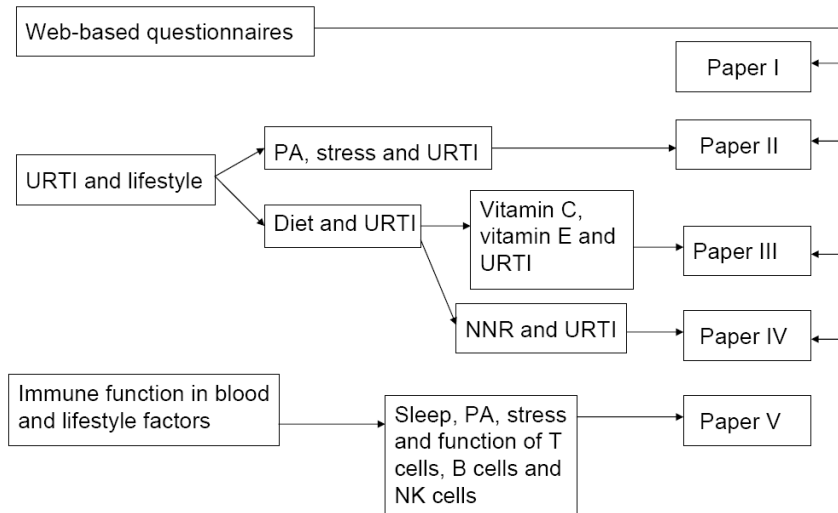


Figure 10. Aims of studies included in thesis.

6 SUBJECTS AND METHODS

6.1 SETTINGS

The papers included in this thesis are based on three different study populations:

- The FAS study (Paper I)
- The LIME study (Papers II-IV)
- The LIM study (Paper V)

Table 2. Descriptive information of study populations included in thesis.

	Pre-FAS pilot I	Pre-FAS pilot II	The FAS study	The LIME study	The LIM study
Paper	-	-	Paper I	Papers II-IV	Paper V
Type of study	Observational	Observational	Observational	Observational	Observational
Study population	Inexperienced computer users recruited from friends and family in the spring and summer of 2002	University students and secondary school students in the spring of 2002	Residents in a middle-sized county in Sweden in the winter of 2002	Residents in a middle-sized county in Sweden in January - May 2004	Self-selected responders to posted ads on a university campus in Sweden in 2003 and 2008
Follow-up time	-	-	8 weeks	15 weeks	3 days
Outcome	-	-	Response-rates	Self-reported URTI	Immunological measures from blood samples
Invited	-	-	875	5000	-
Responded (to 1 st questionnaire)	8	146	483	1805	44
Age range	55-67	16-30	20-59	20-60	20-54

6.2 STUDY DESIGNS

Web-based questionnaires had not been used to collect data in population-based epidemiological studies in 2002. Internet access was fairly high in Sweden at this time, and therefore, we wanted to study the feasibility of using Web-based questionnaires, and investigate which response-rates could be anticipated. For this reason, we conducted a few pilot studies.

6.2.1 Pre-FAS – in preparation for the FAS study

6.2.1.1 Pilot study I - How can a person without computer experience participate in a Web survey?

In epidemiological studies, random selections from a population registry are often used. By using a Web survey as compared to a paper survey, participants who are inexperienced with computers might choose not to participate or are unable to participate. This can be a source of bias, since access to computers and the Internet is not equally distributed over age and work groups (100).

To increase response-rate among inexperienced computer users, written and illustrated instructions were designed. These instructions were then tested among eight participants, three men and five women between ages 55-67, with little or no computer experience who were recruited via friends and family of the persons involved in the project. These eight participants completed the survey on the Web with help from the written instructions, to investigate if the instructions were adequate. Help was provided if the participant needed extra assistance. The instructions on how to complete the survey on the Web were then altered by suggestions from the participants.

Results from the first pilot study conclude the following:

It is helpful for inexperienced computer users if instructions include:

- illustrations of where to find the ENTER and DELETE buttons on the keyboard
- illustrations of how to open and use a Web page (where to write the URL address)
- explanation of how to double-click on a mouse
- explanation of how to enlarge a window
- explanation of how to scroll a page
- explanation of how to close a window

To facilitate completion of a Web questionnaire:

- divide long pages into shorter pages to avoid the need to scroll
- in the URL address, do not use a forward slash (/) or characters that might be difficult to locate on the keyboard
- avoid CAPITAL letters or characters that need the SHIFT key in the password

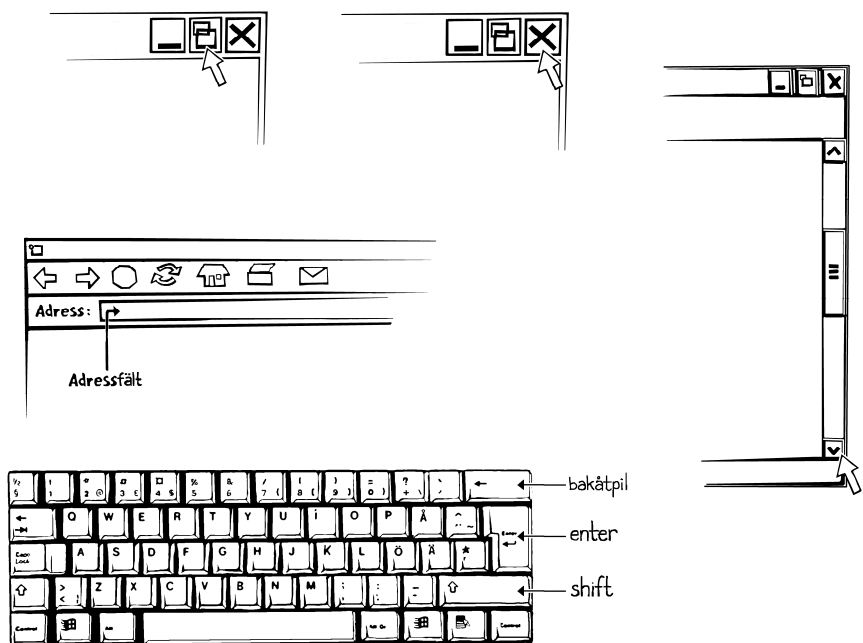


Figure 11. Illustrations on how to use a computer for inexperienced computer users (in Swedish).

A previous study have found that it is important to use a progress indicator so that the participant gets an estimate of how long the questionnaire is to avoid drop-out (101). We implemented a progress indicator in our questionnaire showing current page number and total number of pages.

We found that inexperienced computer users can open a Web browser, go to a homepage and answer a Web survey if given access to a computer, Internet, and adequate instructions. We learned that individual feedback might increase the response-frequency for Web surveys among persons with little or no computer experience. The type of feedback which was found most motivating for future studies was: how to eat to avoid cardiovascular disease, how to eat to loose weight, and feedback on vitamin and mineral intake. Additional suggested motivations for future participation were to give courses on how to use search engines, how to use e-mail, how to pay bills online, and how to do genealogy online.

It takes a great effort for people without computer experience to locate a computer and complete the Web survey. Good instructions on how to locate a computer at a local library or Internet café along with helpful illustrations can make the task easier and individual feedback can give the necessary motivation.

6.2.1.2 Pilot study II - First tests of the Web-questionnaire

The Web questionnaire was tested and evaluated by 61 secondary school students. In addition, another set of 77 secondary school students completed the paper version of the same questionnaire (students were randomly assigned to either Web or paper questionnaire). The average self-estimated time to complete the questionnaire was 17 minutes for paper and 18 minutes for the Web version. However, many (41%) of the students answering the paper questionnaire failed to complete the question on physical activity, whereas all students answering the Web questionnaire completed this question (where summation of time at each activity level was automatic).

6.2.2 The FAS study – the first Web-based cohort study

We randomly selected 875 men and women, aged 20-59 years, living in a middle-sized county, from the Swedish population registry. Participants were thereafter randomized to one of the three versions of the same questionnaires: 1) traditional printed questionnaire, 2) regular Web questionnaire, and, 3) interactive Web questionnaire with personalized feedback.

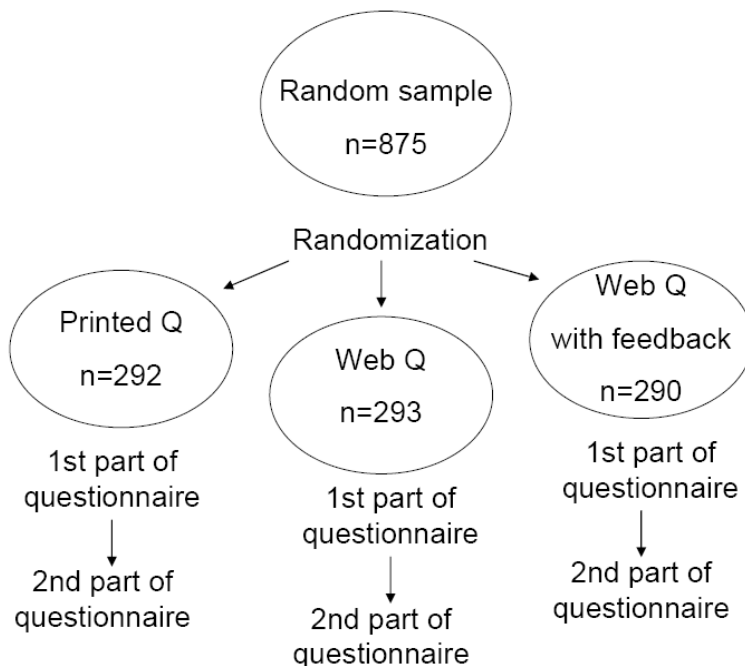


Figure 12. Study design of the FAS study (Paper I).

All participants were sent an invitation letter informing them about the study. After two weeks, one-third of the group was sent a printed questionnaire and two-thirds were sent a letter with information on how to access the Web questionnaire, including details on how to use a Web browser, the URL to the Web questionnaire, and an individual username and password. Information on where to access the Internet in local libraries

and how to use a computer were also given. In addition, half of the Web group (one third of the total) was given the option of personalized feedback about their energy expenditure and body mass index. All non-respondents were reminded after three weeks by a letter, and contacted by phone after an additional three weeks, if necessary. The study was approved by the Ethics committee at Karolinska Institutet.

6.2.3 The LIME study

We randomly selected 5000 men and women from the Swedish population registry who were between 20-60 years old and lived in a Northern middle-sized county. Invitations to participate were sent out via regular paper mail and included information on how to access the Web questionnaire for both experienced and novice computer users. The baseline questionnaire about lifestyle factors also included a question about the participant's e-mail address. Five follow-up questionnaires were sent during the following fifteen weeks (in February, March, early April, late April and May). Every questionnaire included questions on occurrence of URTI during the three preceding weeks. Reminders were sent to non-responders by e-mail 1.5 weeks after each follow-up.

The design of the study is illustrated in Figure 13. Of the 5000 individuals who were invited to participate in the study, 1805 completed the baseline Web questionnaire, and after exclusions, 1509 were eligible for follow-up questionnaires. Exclusions were made for participants who had a URTI at baseline (N=236), lacked an e-mail address (N=17) or chose not to disclose it (N=20). During the study it became evident that participants at one specific workplace had an e-mail server that filtered our e-mails as junk mail. These participants were therefore excluded from the study (N=23) since they could not be invited via e-mail to fill out the follow-up questionnaires. Response rate for each follow-up questionnaire ranged between 83 and 84% (number of responders to that follow-up questionnaire divided by the number eligible for a follow-up questionnaire). In total, 1111 responded (74% of baseline respondents) to all five follow-ups. The study was approved by the Ethics committee at Karolinska Institutet.

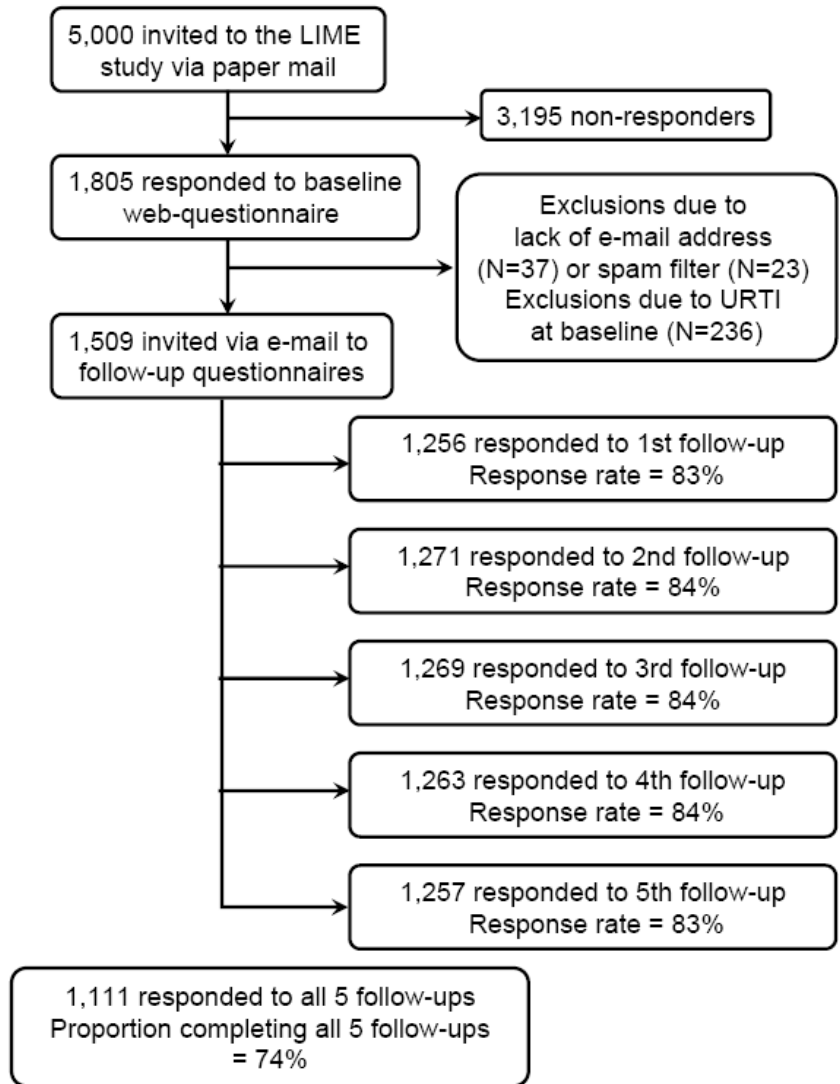


Figure 13. Data collection of the LIME study (Papers II-IV).

6.2.4 The LIM study

In Paper V, 44 healthy self-selected men (n=6) and women (n=38) age range 20-54 responded to posted adds on a university campus and donated blood. Eight participants were excluded as six reported common cold symptoms and two failed to complete the questionnaire resulting in 36 participants (30 women). The mean age was 37.8 years and the majority (74.3%) had a BMI within normal range (table 1a). Of these, 29 donated blood on three consecutive mornings between 8-10 a.m. in November or December, 2003, and seven donated one morning sample in April, 2008. Peripheral blood mononuclear cells (PBMCs) in fresh whole blood were classified and counted in

all participants. Cortisol concentration was analyzed in a sub-group of 32 participants (5 men and 27 women), NKCA in 21 participants (one man and 20 women), and T cell and B cell proliferative responses to antigens in 27 participants (two men and 25 women). Different numbers of participants were used in analyses of immune function since limited man power was available to perform laboratory analyses on fresh blood. All participants were asked to complete a questionnaire on general lifestyle factors (previously described (102)). In addition, the participants filled out a health diary about common cold symptoms, sleep, and physical activity for the 24-hour period prior to each blood draw. The study was approved by the Ethics committee at Karolinska Institutet.

6.3 MEASURES

6.3.1 Self-reported URTI

Self-reported URTI was assessed at baseline and in all five follow-up questionnaires (Papers II-IV). In the follow-up questionnaires, participants were asked if they currently had an infection (cold or influenza) or if they had had a new infection during the last three weeks, or since the last questionnaire. Participants were considered to have a URTI if they answered “yes” to this question. They were instructed not to count a URTI episode twice, even if it crossed over two follow-up periods. Follow-up questions about symptoms were given to all participants who reported a URTI. Seven symptoms were recorded: sore throat, cough, runny nose, headache, malaise, fever, and unspecified symptoms. URTI symptoms that included fever were classified as systemic URTI, whereas cases without fever were considered non-systemic URTI in Paper II.

The influenza season of 2003-2004 in Sweden was of medium intensity. Activity peaked during the last week of December, 2003, and the first week of January, 2004, but declined shortly after and remained low during the rest of the season, which ended in the middle of March (103). Most (94%) of confirmed cases were of the influenza A H3 strain according to Swedish Institute for Infectious Disease Control (SIIDC) (103).

6.3.2 Biological Measures

Fresh whole blood (2 x 5 ml per blood draw) was collected from all 44 participants in the LIM study (Paper V). On the day of the blood draw, tests were set up to measure immune cell numbers, NKCA and 7-day T- and B cell proliferation tests. The tests were performed at SIIDC. In addition, blood samples for plasma collection were centrifuged and stored at -70°C for up to four weeks until they were sent to be analyzed for cortisol concentration at Karolinska University Laboratory.

6.3.2.1 Cell counts

Whole blood was stained with monoclonal antibodies directed against lymphocyte receptors: CD45, CD3, CD4, CD8, CD16/56, and CD19 in TrueCount tubes (BD Biosciences, San Jose, CA, USA). Enumeration is thus given in the same analysis as characterization of the blood cells.

6.3.2.2 T cell and B cell activity

T cell and B cell activity was measured using Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood (FASCIA) (104). 50 µl of fresh whole blood was drawn to heparinized tubes and diluted 1/8 in RPMI-medium (RPMI-

1640) supplemented with penicillin streptomycin (10.000 IU/ml) to tubes containing 100 µl medium for negative control, or 100 µl of any of the following antigens; phytohemagglutinin (PHA), pokeweed mitogen (PWM), influenza fluvirin, or staphylococcus enterotoxin A/B (SEA+B). Tubes were then incubated at 37°C at 7.5% CO₂ for seven days. After the seven day incubation, tubes were stained with anti-CD3 FITC and anti-CD4 PerCP (Becton Dickinson Immunocytometry Systems, Stockholm, Sweden), incubated for 15 minutes, and then a lysing solution was added. Tubes were then centrifuged and washed and cellfix was added (Becton Dickinson). Finally the tubes were stored in the dark for 30 minutes to 24 hours at 4°C until read by FACScalibur using CellQuest software. CD3⁺ CD4⁺ T cell and CD3⁻ B cell proliferative responses to antigens below 20 cells per µl blood were considered background noise and were put to 0.

6.3.2.3 Natural Killer Cell Activity (NKCA)

NKCA was assessed by using a Flow-cytometric Assay of Natural Killer cell Immune response in Activated whole blood (FANKIA) developed at the SIIDC. In this assay, the normally used NK cell sensitive cell line K562 was used as targets. The targets were adjusted to a concentration of 2x10⁵/ml in RPMI+pest+10% FCS i.e. complete culture medium (CM). Three types of reactions were prepared for each subject;

- (1) 50µl whole blood + 50µl CM (single tube)
- (2) 50µl K562 + 50µl CM (duplicate)
- (3) 50µl WB + 50µl K562 (duplicate)

Two hours of incubation at 37°C in 5% CO₂ were followed by staining for 10 minutes with anti-CD71 (FITC) (stains only K562) and thereafter bursting of red blood cells by addition of 500µl BD FACS Lysing Solution. Samples were acquired by flow cytometry within 2h of preparation. A FACScalibur and CellQuestPro software (BD Biosciences) was used for acquisition and the CellQuestPro software was also used for analysis. Cytolysis was detected by measuring loss of K562. The proportion of reduction or percent cytotoxicity was calculated by comparing the number of K562 in the reaction without NK cells (reaction 2) to the number of remaining K562 in the reaction with NK cells (reaction 3). Reaction 1 was used to detect the background, i.e., the number of effector cells in the region for CD71+ K562. These were subtracted from the number of CD71+ cells in the cytotoxic reaction 3 before percent calculation.

6.3.3 Physical Activity

Total physical activity and inactivity was assessed at baseline in the FAS, LIME and LIM study. Participants were asked to estimate the amount of time (hours and minutes) spent on each out of nine activity levels, including sleep, adding up to 24 hours, for a usual day and night. Each activity level was explained by examples of activities corresponding to a MET-value (MET, metabolic equivalent task, multiples of resting metabolic rate) (105, 106). We estimated average MET-hours expended during a 24-hour period by multiplying MET-level by the time spent at each activity level. The MET-values assigned to the nine different levels ranged between 0.9 and 8.0 METs. One MET is equivalent to sitting in a relaxed position. Activities equal to 3-5 MET include walking at a moderate to brisk pace, housework, or playing golf, whereas mowing the lawn with a hand mower or jogging at a slow pace is equal to 6 MET (105, 106). Hours and minutes spent in different activities were automatically summed in the

Web questionnaire and participants were promptly reminded if the total hours and minutes for a typical day did not add up to 24 hours. The method was developed and validated by Lagerros et al. (107), see figure 14. The physical activity questionnaire is available online (108).

How much time a day do you spend doing activities as demanding as:









Level			hours	minutes
A		for example sleeping, lying quietly in bed		
B		for example sitting - bathing, quietly listening to music, watching television, etc.		
C		for example sitting - light office work, knitting, sewing, meetings, etc.		
D		for example making bed, ironing, washing dishes, etc.		
E		for example bowling, driving bus/tractor, automobile repair, dancing waltz/foxtrot, etc.		
F		for example walking briskly, horseback riding, sweeping sidewalk, etc.		
G		for example painting outside house, carrying and stacking wood, skiing downhill, etc.		
H		for example construction work, mowing lawn with hand mower, shoveling snow by hand, etc.		
I		more effort than level H		
			24	00

Figure 14. Questionnaire to assess total physical activity during a typical 24 hour period.

Activity	Intensity	x	Duration	=	MET-hours
Sleep	0.9 MET	x	7 hours	=	0.9 x 7 = 6.3 MET-hours
Brisk walk	4 MET	x	2 hours	=	4 x 2 = 8 MET-hours
Running	8 MET	x	1 hour	=	8 x 1 = 8 MET-hours
				
				Σ 24 hours	Σ MET-hours/day

Table 3. Example of how to calculate MET-hours/day.

In Paper II, physical activity was categorized based on a cross-sectional study using a Danish version of the same physical activity assessment method (109). Total physical activity (MET-hours) was categorized into three levels (<45, 45-<55, ≥55 MET-hours per day), with the lowest group used as reference. In addition, non-vigorous physical activity was defined as activities corresponding to 3-5 MET and vigorous physical activity as activities corresponding to 6 MET or more.

In Paper V, physical activity levels were too low to use the same categorization as in Paper II. A reason for this may be that participants in Paper V (the LIM study) were recruited at a university campus and most had sedentary jobs. In Paper V, physical activity was therefore categorized into low and high general physical activity by grouping individuals below and above the median of 38 MET-hours per day.

6.3.4 Perceived Stress

Stress was assessed by the Perceived Stress Scale (PSS) (41), which is a set of 14 questions of general nature that measures the degree to which life situations are appraised as stressful by the participant. Participants were asked to estimate their stress during the previous month. Questions include “Felt that difficulties were piling up so high that you could not overcome them” and “Been upset because of something that happened unexpectedly”. Answers are given on a 5 grade scale ranging from very often to never. The PSS was developed in the early 80’s by Sheldon Cohen and is widely used to assess perceived stress.

In Paper II, low stress level was defined as having ≤ 23 points (below median) on the PSS and high stress level as having > 23 points (above median). In Paper V, the median of 21 points was used as cut-off for scoring.

6.3.5 Diet

6.3.5.1 Food frequency questionnaire

Diet was assessed by a 96-item validated semi-quantitative food frequency questionnaire, FFQ (110, 111), including vitamin and mineral supplements, measuring usual dietary intake. In Papers II-IV, consumption data from the questionnaire, including portion size, was linked to the Swedish National Food Administration database (112) on energy and nutrient content in various food products in order to calculate the daily mean intake of nutrients (nutrient contents are based on the most commonly consumed form of each food item: fresh / canned / frozen and / or cooked). Spearman correlation coefficients range from 0.38 (iron) to 0.81 (vitamin C) for micronutrients, and are the following for macronutrients: 0.44 (protein), 0.73 (carbohydrates), 0.71 (fiber), 0.70 (total fat), 0.75 (saturated fat), 0.66 (monounsaturated fat), 0.49 (polyunsaturated fatty acids, PUFA) and 0.81 (alcohol) comparing the FFQ to a 7-day food diary (110).

6.3.5.2 Vitamin C and vitamin E from food

In Paper III, vitamin intake from food was calculated as mg/day and categorized into three arbitrary (113) intake groups (vitamin C: <50, 50-150, >150 mg/day for men (range 16-586 mg/day), <100, 100-200, >200 mg/day for women (range 13-592 mg/day), vitamin E: <5, 5-8, >8 mg/day for men and women (range 2-20 mg/day)). The lowest group in each case was used as the reference category. In sub-group analyses, participants with daily or weekly intake of vitamin or multivitamin supplements were

excluded from the analyses of vitamin intake from food under the presumption that supplement intake would mask an effect of vitamin intake from food.

6.3.5.3 Vitamin Supplement Use

Vitamin supplement use was assessed in the baseline questionnaire in the LIME study. Daily, weekly, sporadic or non-use of 13 dietary supplements were recorded. For analysis in Paper III, vitamin C and vitamin E supplement use was categorized into two categories; daily or weekly use, and non-use. Sporadic users were excluded from analyses on supplement use. In sub-group analyses, participants with a high vitamin intake from food were excluded from analyses since a high intake from food could potentially blunt the effect of vitamin supplement use.

6.3.5.4 Dietary Intake of Food Items Rich in Vitamin C

To analyze the effect of eating food products rich in vitamin C in Paper III (rather than vitamin C per se, expressed as mg/day), the number of servings of food items rich in vitamin C was summed up. A serving of a food item rich in vitamin C was defined as a food product with a concentration of at least 200 mg vitamin C per 100 gram of product (vitamin concentration is for boiled or raw product depending on the food item). The products included in the analysis were bell pepper, citrus fruits, broccoli, Brussels sprouts, cabbage, cauliflower, vegetable mix, and berries. The number of servings was categorized into three arbitrary (113) intake groups (<30, 30-60, >60 servings per month), with the lowest frequency used as the reference category.

6.3.5.5 Categorization of NNR adherence

The NNR include recommendations on macronutrients as a percentage of total energy intake, intake of fiber and salt, as well as recommended daily intake of vitamins and minerals and recommendations on physical activity (75).

For the main analysis in Paper IV, the NNR was divided into six groups of individual recommendations; intake from 1) macronutrients (e.g. total fat, protein and carbohydrates), 2) micronutrients (vitamins and minerals), 3) sodium, 4) alcohol, 5) fiber, and 6) physical activity. For each major NNR group, every individual recommendation variable was graded on a continuous 0-1 scale as follows: 1 point was given to intakes within the NNR; 0 point was given to intakes below a defined lowest value and/or above a highest value (for nutrient variables, the median of the ten lowest and/or ten highest intakes among the study population was used, and for physical activity, <30 min/day was used as the lower value); and a relative score of 0-1 points was awarded for intakes or activity levels in between recommendation level and the above defined highest and lowest values according to the following calculation (where Y is the new adherence variable and X is the intake or activity level):

For lower limits, Y varies from 0 to 1: $Y = (X - \text{lowest value}) / (\text{lower NNR cut-point} - \text{lowest value})$

For upper limits, Y varies from 1 to 0: $Y = 1 - ((X - \text{higher NNR cut-point}) / (\text{highest value} - \text{higher NNR cut-point}))$

Summation of the scores of individual recommendations into groups of recommendation (e.g. fat, carbohydrates, vitamins, and minerals) was made by summing up the individual scores and dividing the sum by the number of individual

recommendations included in the group; in that way equal weight was given to each group in the final adherence score. Sodium, alcohol, fiber, and physical activity were kept as individual recommendations. Scores from the six recommendation groups were then summarized into a total score ranging from 0 to 6 points for each person, and divided into three groups of adherence using arbitrary cut-points; <4.5 points for low adherence (range 0.17-4.49, median 4.10), 4.5-5.5 points (median 4.99) for medium adherence and >5.5 (range 5.50-5.83, median 5.60) points for high adherence. The group with low adherence was considered as the reference group in the subsequent analysis.

Moreover, three additional scoring models were tested in order to evaluate the effect of scoring *per se*. The first alternative scoring model differs from the initial model with regards to giving more weight to the score for vitamins and minerals. The second scoring model differs from the initial model by giving more weight to the scores for vitamins, minerals and individual macronutrients. Finally, the third alternative scoring model differs from the initial model by excluding recommendations for total fat intake, monounsaturated fat intake, PUFA intake, protein intake and carbohydrate intake and by only including recommendations that are open-ended (no closed intervals), (e.g. ≤ 10 % of energy from saturated fat). This way, measurement error of the FFQ would have less influence on the results.

6.3.6 Sleep

In Paper V, we assessed sleep duration the night before blood draw (prior sleep) as well as general time spent sleeping and resting (long-term sleep/rest). Prior sleep was reported on the morning of each blood draw, and was later categorized into short prior sleep (<7h, n=17 assessments), normal prior sleep (7-9h, n=65 assessments) and long prior sleep (>9h, n=1 assessment) following Gallicchio et al. (114). Only one participant was categorized as having long prior sleep on one night, and data on long prior sleep was therefore excluded from the sleep analyses.

A questionnaire about general sleep and rest (time spent sleeping and/or resting in bed) during a typical 24-hour period (to investigate long-term effects of sleep) was reported at the time of the first blood draw. General sleep/rest was categorized as short general sleep/rest (<7.5h, n=10 participants), normal general sleep/rest (7.5-9.5h, n=25 participants), and long general sleep/rest (>9.5h n=1 participant), the last being excluded. The cut-offs for prior and general sleep/rest were adjusted to accommodate the fact that general sleep/rest included time resting in bed while prior sleep measured time asleep only.

6.4 STATISTICAL ANALYSES

In Paper I, simple descriptive analyses were used to calculate response rates and continuation rates. Poisson regression was used in Papers II, III, and IV. We used stratified analysis as a primary approach to assess and control confounding. Spline regression was used for graphical purposes in Papers II, III, and IV. For Paper V, a linear regression model and a mixed-effects linear regression model were used.

6.4.1 Papers II, III, and IV

6.4.1.1 Incidence rates tables

In Papers II and III, raw data is presented in tables by main exposure, age, sex and URTI risk. We standardized for age and sex using the age-sex person-time distribution

of the low physical activity (Paper II) or vitamin C group (Paper III) as the standard (115). These tables show results based on simple computations and the purpose is to show the reader what the data looks like. Additional confounding factors, such as BMI, contact with children, and season have not been adjusted for in these tables.

6.4.1.2 Poisson regression

In Papers II, III, and IV, we divided the number of reported URTI by person-time at risk to get incidence rates. We also estimated incidence rate ratios (IRR) and 95% confidence intervals (CI) using Poisson regression models to control for age and sex along with other confounding factors. Disease-free participants contributed three weeks of time at risk for each 3-week follow-up period. A participant with no reported URTI could therefore contribute up to five 3-week periods of time at risk, a total of 15 person-weeks. Since we did not know exactly when a URTI occurred or how long it lasted during the three week period, we assigned participants who reported a URTI 1.5 weeks of risk time out of the 3-week follow-up period.

Poisson distribution is the most widely used distribution for modeling counts. Poisson regression is based on Poisson distribution and uses maximum-likelihood estimation (based on specific assumptions how Y varies when X is fixed). Poisson regression can be used for either count data or rates (count by a specified time period).

The Poisson distribution assumes that:

- 1) every event is clearly defined
- 2) every event is independent of the previous event
- 3) the rate of events does not change within each time interval
- 4) each event is not counted more than once

URTI is clearly defined in the LIME study by self-report, fulfilling assumption number 1. However, it could be argued that URTIs are not completely independent events. Having a URTI could possibly increase or decrease the risk of getting another URTI and thus potentially violate assumption number 2. To investigate this, we fitted a generalized estimating equations (GEE) model that accounts for repeated events in the model (see separate section on GEE below). In addition, a negative binomial model was fitted which does assume that every event is independent, but results were very similar to the Poisson model in all analyses. Each time interval was three weeks and we assume that the risk of URTI does not change greatly within each three-week period. Therefore assumption number 3 was probably not violated. We asked participants not to count a URTI more than once, which complies with assumption number 4.

6.4.1.3 Goodness of fit

One way to evaluate goodness of fit in Poisson regression is to study deviance or chi-square divided by degrees of freedom (DF) to see if there is overdispersion or underdispersion. Overdispersion, which is more common, is the situation in which there is excess variation around fitted values. Underdispersion describes the situation in which there is lower variation than expected. There were little evidence of overdispersion or underdispersion in the data (deviance/ DF= 0.82 and chi-square/ DF = 1.62) in Paper II, nor in Paper III or IV (data not shown).

6.4.1.4 Generalized estimating equations

GEE is used to take into account lack of independence among subsets of the basic units of observation. GEE works by adding a correlation matrix to the regression model. An unstructured correlation matrix is the least restrictive matrix and allows for every correlation to be different (116). Since there may be correlation in the data due to repeated measurements on the same individual in Papers II-IV, GEE with an unstructured correlation matrix was added to the Poisson model to account for this. However, very little correlation was found in the data and GEE was therefore not used in the final analyses.

6.4.1.5 Spline regression

Since the exposures were continuous in Papers II-IV, we were able to use multivariable spline regression models to illustrate the associations. An advantage of spline regression is that the exposure can remain continuous and that the spline graphs are easy to understand and interpret. However, certain set-points (knots) are arbitrarily chosen and placement of these knots can have a large impact of the appearance of the spline. The more knots used, the more irregularly shaped the spline becomes. Fewer knots produce a smoother curve. This behavior makes goodness of fit testing very easy: if increasing the number of knots systematically and radically changes the appearance of the spline graph, the fit is not good. Conversely, if the change is unsystematic and small, the fit is good (117).

In Papers II-IV, the β was estimated using Poisson regression and 6 knots were decided a priori to be placed at quintile cut-offs, and at minimum and maximum values within a set range. Additional splines were also made to see how different number of knots and placement of knots affected the graphs. All spline graphs in Papers II-IV seemed to have a good fit.

6.4.1.6 Confounder identification

Identification of confounders can be done in two ways, backward deletion or forward addition. The most common approach, backward deletion, is done by including all confounders of interest in the model and then excluding one at a time. Usually, a confounder is kept in the model if the effect estimate changes more than 10%. This approach for confounder identification was used for Papers II and IV. However, for Paper III, a large number of nutrients were studied, and since some of these nutrients can be highly correlated, putting all of them in the same model might introduce collinearity. Therefore, in Paper IV, forward addition was used instead (57).

6.4.1.7 Effect measure modification

Effect measure modification occurs when the association of the exposure and the outcome changes depending on a third variable, an effect modifier. Effect measure modification is scale dependent and can be assessed on the absolute (additive) scale (difference in rate depending on a third variable) or the relative (multiplicative) scale (difference in rate ratio depending on a third variable). In addition to exploring the presence of effect measure modification, it is also of great importance to explore how this effect measure modification appears. This was done in Paper II by using spline curves to see how the relationship between exposure (physical activity) and outcome (URTI) is modified by an effect measure modifier (stress) (118).

Additional analyses on effect measure modification on both the additive and relative scales were made for Paper II, although these were not presented in the paper. Effect measure modification on the relative scale was analyzed by adding an interaction term (between the exposure and the suggested effect modifier) to the Poisson model and the statistical significance of the interaction term was tested by the likelihood ratio test. Stress was found to be an effect modifier on physical activity and URTI at a significance level of $p=0.005$ when physical activity was categorized as <45 , $45-55$, >55 MET-h/day and stress as ≤ 23 , >23 (median cut-off). To see if categorization of physical activity greatly changed the results, additional categorizations were tried (for example quartile cut-offs). The p-values for effect measure modification for these additional physical activity categorizations ranged from $p=0.003$ to $p=0.06$ ($p=0.02$ for quartile cut-offs).

6.4.2 Paper V

6.4.2.1 Linear regression

For validity of statistical inference (CIs) using linear regression in small samples, the additive error term should be normally distributed with constant variance (homoscedasticity). We used diagnostic plots of the residuals to assess these assumptions for our models: normal quantile plots were used to check for normal distribution. For a number of outcomes, we found some curvature in the quantile plots, but after log-transforming all variables, we found that the residuals were throughout closer to normality. We checked for homoscedasticity by plotting the residuals from the log-transformed regression models against the predicted values; the spread of the residuals appeared to be constant, indicating constant variances. The estimated coefficients (betas) and CIs from the log-transformed models were exponentiated, translating in effect the additive models for the log-transformed data into multiplicative models on the original scale. The exponentiated coefficients estimate the multiplicative change in the outcome variable when the exposure is increased by one unit. These estimated factors can then be reported either directly (e.g. as factor 1.3) or as percentage change (i.e. +30% in this example) (119).

The R^2 value from the linear regression model gives an indication of the fit of the model. The R^2 value (e.g. 0.42) from a linear regression indicates how much (e.g. 42%) of the variance in Y that can be attributed to the model. The remainder (here: 58%) is assumed to be due to biological variation or measurement error. For samples with large biological variation, as in our data, a lower R^2 is expected even if a linear model is a good fit. The original models that indicated an association between lifestyle and immune factor had adjusted R^2 values ranging from 0.23-0.40 which were consistently increased after log-transformation of variables with adjusted R^2 values ranging from 0.26-0.45, indicating that log-transformations of variables gave a slightly better fit.

In Paper V, a within-subject design was used for analyses on prior sleep, using immune and lifestyle data from three consecutive mornings (repeated measures on the same individual). A mixed-effects linear regression model was used to account for between-person variation, with a random intercept accounting for person-to-person variation. Total time awake before blood draw was adjusted for as a confounder.

6.4.2.2 Standard error and standard deviation

In Paper V, we used standard deviations (SD) in the descriptive tables and standard error (SE) bars in figure 1 and 2. SD is used to illustrate variability in the data and is not affected by sample size. The SE is used to illustrate the uncertainty of the estimate of mean and is affected by sample size (120).

7 RESULTS

7.1 FEASIBILITY OF WEB QUESTIONNAIRES

Response rate for the first section of the questionnaire was higher among those responding to printed questionnaires (64%, table 4) than among those responding to Web questionnaires. However, compliance (willingness to answer the second part of the questionnaire) was higher for the Web questionnaires than for the printed questionnaire. Fifty-three percent of those who completed the general section of the paper questionnaire went on to complete the dietary part, compared with 58% of those responding to the Web questionnaire and 64% of those who were given the interactive Web questionnaire. Thus, the total response rate for the second part of the questionnaire was similar for the three groups (34%, 29 and 32% respectively) (102).

There were no differences in response rate for the three questionnaires by age, BMI, and current smoking, and only small differences by sex, education, and food habits. The self-reported time spent answering the questionnaires did not differ between the groups. In general, those responding to the Web questionnaires were also more frequent users of the Internet, and more able to arrange for privacy when using the Internet, assuring that no one was watching the screen.

Of those who completed the Web questionnaires, three participants had never before used the Internet and 18 reported using the Internet very rarely (less than once / month).

Table 4. Response rates for each group by type of questionnaires; printed questionnaire, basic Web questionnaire, and Web questionnaire with personalized feedback (102).

	Printed Questionnaire (n = 292)	Web Questionnaire (n = 293)	Web Questionnaire With feedback (n = 290)
Number of subjects answering the first section of the questionnaire*	188	149	146
Response rate; % (95% CI)	64 (59-70)	51 (45-57)	50 (45-56)
Number of subjects continuing with the second section of the questionnaire†	100	86	93
Continuation rate; % (95% CI)‡	53 (46-60)	58 (50-66)	64 (56-71)
Total response rate for the second section; % (95% CI)	34 (29-40)	29 (24-35)	32 (27-37)

*The general section of the questionnaire about lifestyle factors (the first part of the questionnaire).

†The food frequency questionnaire (the second part of the questionnaire).

‡Only participants answering the general section of the questionnaire were invited to complete the food frequency questionnaire.

7.2 PHYSICAL ACTIVITY, STRESS, AND URTI

In the LIME study, we found that there were more infections during the winter than in the spring, 23% of the participants reported a URTI in February compared with only 14% in May.

Baseline characteristics of the participants in Paper II show that women and those with higher levels of education were more likely to report lower levels of physical activity. The age and sex standardized IRR of URTI by level of physical activity are shown in table 5, and indicate that high levels of physical activity are associated with a reduced IRR of URTI among both men and women in most age-groups. Results also show that high levels of total physical activity (≥ 55 MET-hours per day) were associated with an 18% reduced risk of URTI compared with low levels of physical activity (IRR 0.82, 95% CI: 0.69-0.98). For non-vigorous physical activity (equivalent to walking or doing housework), a similar association was found (IRR 0.89, 95 % CI: 0.79-1.01) that was slightly weaker but analogous to that found for vigorous physical activity (equivalent to jogging; IRR 0.86, 95% CI: 0.75-0.98) (table 6). When separating URTI into non-systemic (no fever) and systemic symptoms (including fever), the strong protective effect of physical activity remained for URTI with non-systemic symptoms, which constitutes the majority of the cases (76%), whereas little effect of physical activity was seen for URTI with systemic symptoms (121).

An additional analysis was made to reduce the influence of repeated events in the same person, where only the first URTI per person was included, but the results were similar to findings from the main analysis. In addition, because physical activity habits might vary with season, an analysis was conducted in which only URTI cases occurring during February, March and early April were included, but the results were unchanged.

The Poisson regression model assumes that each new outcome is independent of the previous outcome, and it could be argued that having a URTI can make you more susceptible to contracting another URTI which would make the Poisson model assumption of independence untrue. Therefore, a negative binomial regression model was also fitted. Results were very similar to the Poisson regression which suggests that URTI episodes were independent events in this study population.

No effect on the risk of URTI for perceived stress was seen in the whole group (age and sex adjusted IRR 1.05, 95% CI: 0.94-1.18). However, results indicate that the inverse association of physical activity on URTI risk was stronger in the group with high perceived stress (IRR 0.58, 95% CI: 0.43-0.78) than in the group with low stress levels (IRR 1.03, 95% CI: 0.83-1.28) (table 7). No indication of any important effect measure modification on physical activity by age, body mass index, smoking, month or contact with children was found (121).

Table 5. Incidence rates for URTI by age, sex, and level of physical activity (IRRs are standardized for age and sex) (121).

Age	Low physical activity (<45 MET-hours/day)*			Medium physical activity (45-55 MET-hours/day)			High physical activity (≥ 55 MET-hours/day)		
	No. of cases	Person-weeks	Rate/1000 person-weeks	No. of cases	Person-weeks	Rate/1000 person-weeks	No. of cases	Person-weeks	Rate/1000 person-weeks
Men									
20-29	72	942	76.4	62	675	91.9	34	414	82.1
30-39	85	1018.5	83.5	37	490.5	75.4	19	334.5	56.8
40-49	41	670.5	61.1	25	457.5	54.6	22	459	47.9
50-60	49	949.5	51.6	21	484.5	43.3	12	510	23.5
Total	247	3580.5	69.0	145	2107.5	68.8	87	1717.5	50.7
Women									
20-29	183	1852.5	98.8	64	831	77.0	33	394.5	83.7
30-39	137	1396.5	98.1	37	685.5	54.0	26	345	75.4
40-49	61	988.5	61.7	24	357	67.2	22	345	63.8
50-60	55	1237.5	44.4	24	549	43.7	12	285	42.1
Total	436	5475	79.6	149	2422.5	61.5	93	1369.5	67.9
Standardized for age and sex [†]		IRR	95% CI		IRR	95% CI		IRR	95% CI
		1.00			0.85	0.74-0.98		0.83	0.69-0.98

*Reference category.

[†]Person-time for the low physical activity group was used as the standard.

Table 6. Incidence rate ratio of URTI by level of physical activity, adjusted for twelve confounding factors (121).

Physical activity (MET-hours/day)	No. of cases	Person-weeks	Adjusted for age and sex		Multivariable model	
			IRR	95% CI	IRR*	95% CI
All URTI cases						
Low (<45) [†]	648	8646	1.00		1.00	
Medium (45-55)	273	4267.5	0.86	0.75-0.98	0.84	0.73-0.97
High (≥55)	169	2866.5	0.81	0.69-0.96	0.82	0.69-0.98
Nonsystemic URTI cases [‡]						
Low (<45) [†]	502	8865	1.00		1.00	
Medium (45-55)	204	4371	0.85	0.72-0.99	0.82	0.69-0.97
High (≥55)	121	2938.5	0.75	0.62-0.91	0.75	0.61-0.93
Systemic URTI cases ^{††}						
Low (<45) [†]	146	9399	1.00		1.00	
Medium (45-55)	69	4573.5	0.90	0.68-1.20	0.94	0.70-1.26
High (≥55)	48	3048	1.02	0.74-1.41	1.10	0.78-1.55

*Adjusted for age (20-29, 30-39, 40-49, and 50-60), sex, body mass index (low, normal, overweight, and obese), asthma (yes/no), weakened immune system (yes/no), perceived stress (below and above median), contact with children at home or work (yes/no), regular contact with large crowds at leisure time (yes/no), education level (secondary school or less and university), intake of vitamin D, vitamin E, selenium (energy-adjusted intakes in four categories) and month (February to May).

[†]Reference category.

[‡]Only including URTI cases with no fever.

^{††}Only including URTI cases with fever.

Table 7. Incidence rate ratio for URTI by levels of physical activity and perceived stress (121).

Physical activity (MET-hours/day)	Low perceived stress (below median)			High perceived stress (above median)				
	No. of cases	Person-weeks	IRR*	95% CI	No. of cases	Person-weeks	IRR*	95% CI
All URTI cases								
Low (<45) [†]	301	4408.5	1.00		347	4237.5	1.00	
Medium (45-55)	149	2371.5	0.89	0.73-1.09	124	1896	0.80	0.66-0.99
High (≥55)	119	1690.5	1.03	0.83-1.28	50	1176	0.58	0.43-0.78
Men								
Low (<45) [†]	188	3375	1.00		143	2281.5	1.00	
Medium (45-55)	114	1947	1.05	0.83-1.34	78	1287	0.95	0.72-1.27
High (≥55)	88	1602	1.01	0.78-1.32	22	999	0.37	0.24-0.59
Women								
Low (<45) [†]	245	3733.5	1.00		354	4566	1.00	
Medium (45-55)	92	1905	0.70	0.55-0.90	88	1761	0.66	0.52-0.84
High (≥55)	85	1132.5	1.19	0.92-1.54	49	883.5	0.78	0.57-1.05

I *IRR were adjusted for age (20-29, 30-39, 40-49, and 50-60), sex, body mass index (low, normal, overweight, and obese), asthma (yes/no), weakened immune system (yes/no), contact with children at home or work (yes/no), regular contact with large crowds at leisure time (yes/no), education level (secondary school or less and university), intake of vitamin D, vitamin E, selenium (energy-adjusted intakes in four categories) and month (February to May).

[†]Reference category.

The shape of the dose-response relationship between physical activity and stress on URTI risk was also examined by using spline regression. Results were similar for both the categorical and spline analyses. Figure 15 shows smoothed incidence rates of URTI for high and low stress groups over MET-hours/day for all participants (A), and for men (B) and women (C), respectively. For the graph showing all participants, high levels of physical activity appeared protective for those experiencing either low or high levels of perceived stress, but the protective effect was much greater for those experiencing high levels of perceived stress. For low stress the apparent protective effect of physical activity (shown as a decline in the rate) comes with lower levels of physical activity and then levels out. For high stress, the protective effect of physical activity comes with higher levels of physical activity but then increases more steadily. For men, the protective effect of physical activity appears to be very small, if any, among those experiencing low levels of perceived stress, and much greater among those experiencing high levels of perceived stress. For women, the difference in stress levels does not appear to influence greatly the effect of physical activity. There is considerable overlap in the 95% confidence bands for high and low stress, so our inference about trends is tentative and limited to a broad characterization.

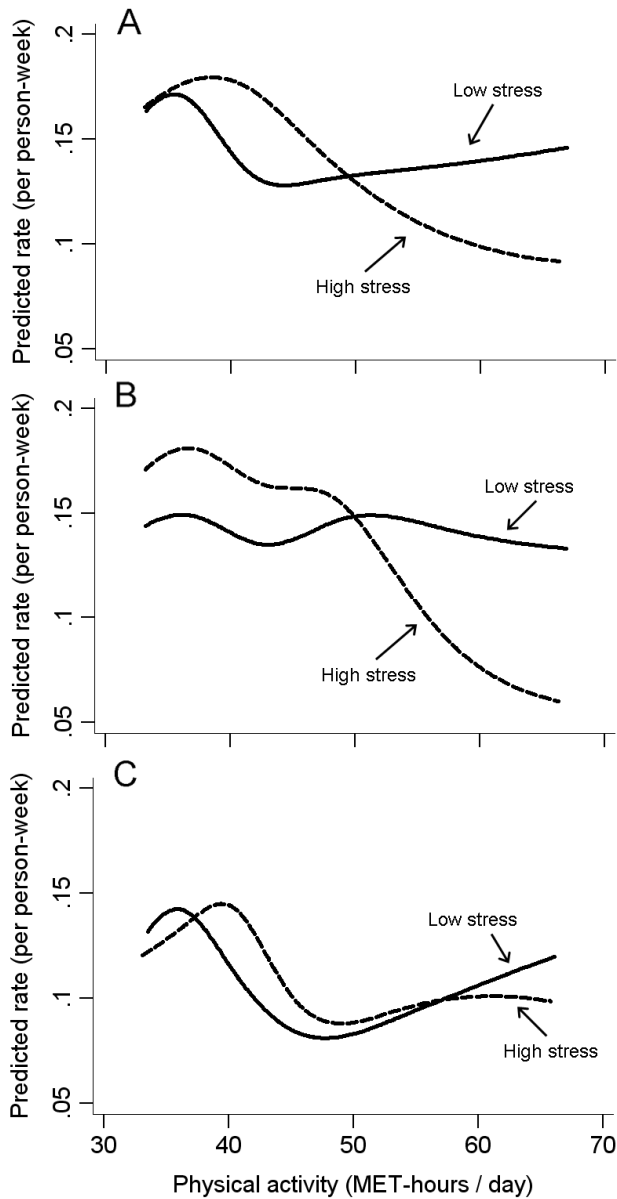


Figure 15. Smoothed incidence rates of self-reported URTI (Upper Respiratory Tract Infection) for physical activity by level of stress, for all (A), for men (B), and for women (C). The smoothed rates of URTI for high and low stress over varying levels of MET-hours/day (Metabolic Equivalent Task) are from a spline model. The rate is predicted at the reference level for all other covariates in the model; young (age=20-29), women (for figure A), with normal body mass index, no asthma, no weakened immune system, no contact with children at home or work, no regular contact with large crowds at leisure time, low-medium intake of vitamin D, vitamin E, second lowest selenium intake categories after adjusted for energy intake, and February as the reference point for monthly effects. The graph has been truncated to show only MET-hours/day values between 33-67 (121).

7.3 VITAMIN C, VITAMIN E, AND URTI

Baseline characteristics show that older participants and those with a higher energy intake, a higher body mass index, a higher physical activity level, or lower perceived stress were to some extent more likely to report a higher intake of vitamin C from food. In general, the intake of vitamin C from food was higher among women than among men. The mean daily vitamin C intake was 134.5 mg for women and 88.3 mg for men, and 48% of the men and 71% of the women reached the recommended intake by the Nordic Nutrition Recommendations of 75 mg vitamin C/day (75).

Vitamin C and multivitamin supplement use were slightly more common among women than men and among participants aged 20-39 than aged 40-60. The incidence rate for URTI decreased with increasing age for both men and women. The standardized IRR was 0.66 (95% CI 0.48-0.90) for women, whereas no association was seen among men. Among women, the overall effect estimate for vitamin C intake from food was 0.69 (95% CI 0.49-0.98), comparing the highest level of intake (>200 mg/day) with the lowest (<100 mg/day), which is concurrent with the IRR for frequent consumption of food items rich in vitamin C, 0.63 (95% CI 0.48-0.83). No association was found for intake of vitamin C from food on URTI risk among men. The overall effect estimate for vitamin C intake from food was 1.16 (95% CI 0.79-1.70) when comparing the highest level of intake (>150 mg/day) with the lowest (<50 mg/day).

To disentangle the effect of antioxidants from food from the effect of antioxidants from supplements, we analyzed the effect of antioxidants from food among non-users of vitamin supplements. Table 8 shows IRRs for dietary intake of vitamin C and vitamin E from the Poisson model adjusting for age and other confounders among those with no daily or weekly vitamin supplement intakes. High dietary intake of vitamin C (>200 mg/day) from food was associated with a 49% reduced risk of URTI compared with low intake levels (<100 mg/day) of dietary vitamin C (IRR 0.51, 95% CI 0.33-0.80) among women reporting no use of vitamin C supplements. For frequent consumption of food items rich in vitamin C, a 45% reduced risk of URTI was found for high consumption (>60 times/month) as compared to low consumption (<30 items/month) in women (IRR 0.55 (95% CI 0.39-0.77), but not among men (IRR 0.99, 95% CI 0.65-1.51). Nor were high dietary intake of >150 mg/day of vitamin C associated with a reduced risk of URTI (IRR 1.25, 95% CI 0.76-2.05) among men reporting no vitamin C supplement use. We saw little association between a high dietary intake of vitamin E and URTI risk.

Table 8. Dietary intake of vitamin C and vitamin E from food and risk of URTI among those with no daily or weekly intake of corresponding vitamin or multivitamin supplement use.

	Cases	Person-Weeks	IRR	95% CI	IRR ^{a-d}	95% CI
					Adjusted for Age	Multivariable model
Dietary intake of vitamin C (mg/day)						
Women						
<100 ^e	228	2922	1.00	-	1.00	-
100-200	165	2161.5	1.06	0.87-1.30	0.94	0.72-1.23
>200	63	1273.5	0.72	0.54-0.96	0.51	0.33-0.80
Men						
<50 ^e	91	1489.5	1.00	-	1.00	-
50-150	228	3567	1.18	0.93-1.51	1.27	0.96-1.68
>150	30	543	1.15	0.76-1.75	1.25	0.76-2.05
No. of vitamin C-rich food items ^f / month						
Women						
Low intake (<30) ^e	140	1629	1.00	-	1.00	-
Medium (30-60)	188	2523	0.92	0.74-1.15	0.81	0.63-1.04
High (>60)	129	2218.5	0.76	0.59-0.97	0.55	0.39-0.77
Men						
Low intake (<30) ^e	168	2597	1.00	-	1.00	-
Medium (30-60)	135	2086.5	1.16	0.92-1.46	1.16	0.90-1.49
High (>60)	46	816	1.02	0.74-1.42	0.99	0.65-1.51
Dietary intake of vitamin E (mg/day)						
Women						
<5 ^e	148	2058	1.00	-	1.00	-
5-8	238	3378	1.03	0.84-1.27	0.99	0.72-1.35
≥8	71	1039.5	1.05	0.78-1.39	1.36	0.83-2.25
Men						
<5 ^e	94	1521	1.00	-	1.00	-
5-8	196	3177	0.98	0.76-1.25	0.96	0.66-1.39
≥8	60	987	1.06	0.77-1.47	0.96	0.57-1.62

^aIRR for vitamin C and vitamin E from diet and vitamin C-rich food items for men and women were adjusted for age (20-29, 30-39, 40-49, and 50-60), energy intake (in four categories), body mass index (low, normal, overweight, and obese), weakened immune system (yes/no), asthma (yes/no), perceived stress (below and above median), smoking (current, previous, never), education level (secondary school or less and university), vitamin E (in four intake groups), zinc (in four intake groups), and month (February to May).

^bIRR for vitamin C from diet for women was also adjusted for folate intake (in four intake groups).

^cIRR for vitamin C-rich food items for men and women were also adjusted for contact with small children (yes/no), intake of vitamin E-rich food items (in three intake groups), and β-carotene-rich food items (in three intake groups).

^dIRR for vitamin E for men and women were also adjusted for intakes of vitamin C, β-carotene, folate, and eicosapentaenoic acid (in four intake groups).

^eReference category.

^fVitamin C-rich food items include: bell peppers, citrus fruits, broccoli, Brussels sprouts, cabbage, cauliflower, vegetable mix and berries.

The effect estimate for vitamin C supplement use was 0.69, 95% CI 0.47-1.02 among men, comparing daily or weekly vitamin C supplement use with non-users, and 1.04, 95% CI 0.76-1.42 among women. We saw an protective association of daily or weekly vitamin E supplement use compared with no supplement use among men, IRR 0.56 (95% CI 0.33-0.95).

We used different categorizations for vitamin C from food for women and men, because women had generally higher intake of vitamin C from food. When we used the same categorization for women as for men (<50mg/day, 50-150mg/day, >150mg/day), results were similar. We found slightly weaker associations, but still indicating protection, for among women with zero supplement intake of vitamin C. The IRR for a high intake of vitamin C from food (>150mg/day) was 0.54 (95% CI 0.34-0.88) compared with a low intake (<50mg/day).

Since pollen allergy can mimic a URTI, we excluded the last two follow-ups, which corresponded to pollen season, for those reporting a pollen allergy. The results were similar to the findings from the main analyses: among women reporting no supplemental use of vitamin C, a high intake of vitamin C from food was associated with a decreased risk of URTI, with an IRR 0.45 (95% CI 0.28-0.71) compared with low intake. Among men, however, the IRR was near a null effect, at 1.09 (95% CI 0.65-1.83). When excluding the last two follow-ups among those reporting a pollen allergy, results were also similar to the findings from the main analyses for vitamin supplement use: vitamin C supplement among women IRR 1.02 (95% CI 0.74-1.42), and among men, IRR 0.71 (95% CI 0.47-1.05). Results for vitamin E supplement use among women was: IRR 1.13 (95% CI 0.75-1.69), and among men: IRR 0.58 (95% CI 0.34-0.99).

Because dietary intake might have varied during our study, we also conducted an analysis in which we included only URTI cases occurring during the first three follow-ups. The results showed a stronger association between vitamin C from food and URTI among women reporting no supplemental use. For those with a high intake of vitamin C from food, compared with a low intake, the IRR was 0.35 (95% CI 0.19-0.64). In contrast, for men, the corresponding estimate moved closer to a null effect when restricting to the first three follow-ups.

Other supplements considered for URTI risk were garlic capsules and Echinacea supplements. Intake of these supplements was too infrequent, however, to yield any meaningful results. To avoid confounding from use of these supplements, we excluded all participants who reported use of these supplements and then re-analyzed the data for use of vitamin C and vitamin E supplements. The results remained very similar to the main analyses.

We investigated the shape of the dose-response relationship of vitamin C intake from food on URTI risk by using spline regression as a smoothing method. Results were similar for both the categorical and spline analyses. Figure 16 shows smoothed incidence rates of URTI for all women (A), women with no vitamin C supplement use (B), and all men (C). High intake (>200mg/day) of vitamin C from food appeared protective for women (shown as a decline in the rate) compared with a low intake (<100mg/day) of vitamin C from food.

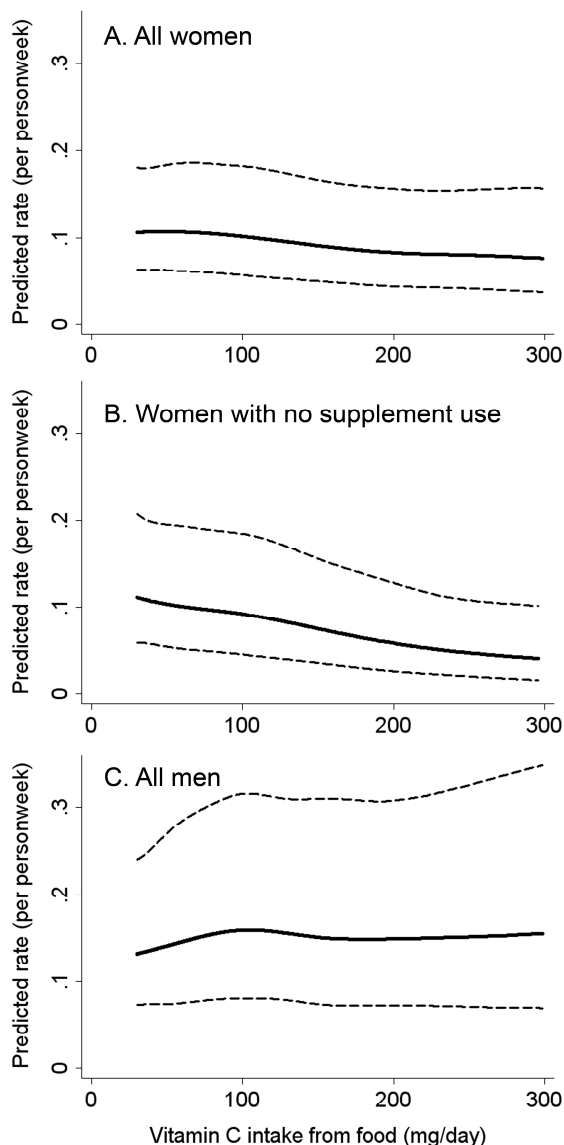


Figure 16. Smoothed incidence rates of URTI for dietary vitamin C intake from food and 95 % Confidence Intervals (dotted lines) for all women (A), women with no vitamin C supplement use (B), and all men (C). The smoothed rates of URTI for vitamin C intake / day are from a spline model. The rate is predicted at the reference level for all other covariates in the model; young (age=20-29), with low-normal body mass index, low perceived stress (below median), no asthma, no weak immune system, never smokers, low education (secondary school or less), low intake of vitamin E, zinc, and folate (folate used among women only), and at February measurement to control for monthly effects. The graph has been truncated to only show vitamin C intake from food between 30-300 mg/day

We found no indication of any large effect measure modification on dietary or supplementary vitamin C or vitamin E intake by age (20-29, 30-39, 40-49, and 50-60), body mass index (low, normal, overweight, and obese), smoking (never, previous, ≤ 10 cigarettes/day, >10 cigarettes/day), asthma (yes/no), fatty fish intake (low, medium, high), or physical activity (low, medium, high).

7.4 NORDIC NUTRITION RECOMMENDATIONS AND URTI

We found only small differences in baseline characteristics among study participants regarding age, sex, body mass index, asthma, and energy intake. Higher adherence to the NNR appeared to be associated with lower perceived stress, lower education level and fewer smokers.

When looking at adherence to each dietary recommendation, we found that PUFA and vitamin D showed the least adherence, 7% of participants were within recommended interval for PUFA, and only 8% reached recommended intake level for vitamin D. Mean intake for PUFA was 3.8 % of the total energy intake (SD 0.8), which is well below recommended intake of 10-12.5% of energy. Observed intakes for vitamin D was also well below recommended intake of 7.5 mcg/d, with an observed mean of 4.2 mcg/d (SD 2.2). Participants also scored lower for α -tocopherol (13%), and selenium (16%), where the mean intake was 6.4 mg/d (SD 2.3) and 33.3 mcg/d (SD 13.8), respectively, much lower than the recommended intakes of 10 mg/d (for men) and 50 mcg/d (for men), respectively (122).

We found that 50% of study participants had medium adherence (4.5-5.5 points) and 13 % had high adherence (>5.5 points) to the NNR according to the initial scoring model. Using the initial scoring model, high adherence to the NNR (>5.5 points) was not associated with a lower risk of URTI compared with low adherence (<4.5 points) (IRR 0.89, 95% CI 0.73-1.08). In order to evaluate the effect of different scoring models for adherence, we tested three additional scoring models. However, the results did not change much. This may partially be explained by the correlation coefficients between the initial scoring models and the alternative scoring models being relatively high, as expected, ranging from 0.75 - 0.85 (122).

Table 9. Initial and alternative scoring models of adherence to the Nordic Nutrition Recommendations (NNR) and incidence rate ratio (IRR) for URTI (122).

	Cases	Adjusted for age and sex			Multivariable model	
		Person-weeks	IRR	95% CI	IRR*	95% CI
Adherence to NNR, initial scoring model [†] (score 0-6)						
Low (<4.5 points)	242	3285	1.00		1.00	
Medium (4.5-5.5 points)	686	9867	0.91	0.80-1.03	0.88	0.77-1.01
High (>5.5 points)	206	3246	0.93	0.78-1.12	0.89	0.73-1.08
Adherence to NNR, alternative scoring model 1 [‡] (score 0-7)						
Low (<5.0 points)	155	2293.5	1.00		1.00	
Medium (5.0-6.0 points)	518	7038	1.02	0.88-1.18	0.98	0.83-1.15
High (>6.0 points)	461	7066.5	0.97	0.83-1.14	0.91	0.76-1.09
Adherence to NNR, alternative scoring model 2 [§] (score 0-9)						
Low (<7.0 points)	319	4456.5	1.00		1.00	
Medium (7.0-8.0 points)	590	8451	0.92	0.81-1.04	0.88	0.77-1.01
High (>8.0 points)	225	3490.5	0.96	0.81-1.13	0.89	0.74-1.08
Adherence to NNR, alternative scoring model 3 (score 0-8) (only including macronutrient recommendations with open-ended cut-offs)						
Low (<6.0 points)	144	2043	1.00		1.00	
Medium (6.0-7.0 points)	472	6393	1.03	0.89-1.20	0.98	0.83-1.16
High (>7.0 points)	518	7962	0.98	0.84-1.15	0.91	0.76-1.09

*IRR for all adherence scores were adjusted for age (20-29, 30-39, 40-49, and 50-60), sex, energy intake (in four categories), body mass index (low, normal, overweight, and obese), weakened immune system (yes/no), asthma (yes/no), perceived stress (below and above median), education level (secondary school or less and university), smoking (daily/less frequent/previous/never), and month (February to May).

[†]Scoring was calculated by awarding one point for adherence to the following recommendation sub-groups in NNR; physical activity (1p), fiber intake (1p), sodium intake (1p), alcohol intake (1p), mineral and vitamin intake (1p), and macronutrient intake (1p).

[‡]Scoring was calculated by awarding one point for adherence to the following recommendation sub-groups in NNR; physical activity (1p), fiber intake

(1p), sodium intake (1p), alcohol intake (1p), mineral (1p) and vitamin intake (1p), and macronutrient intakes (1p).

§Scoring was calculated by awarding one point for adherence to the following recommendation sub-groups in NNR; physical activity (1p), fiber intake (1p), sodium intake (1p), alcohol intake (1p), mineral (1p) and vitamin intake (1p), and one point to each sub-group in macronutrient recommendations; carbohydrate and sugar intake (1p), total fat intake, saturated fat intake, essential fat intake, polyunsaturated fatty acids intake, monounsaturated fat intake (1p), and protein intake (1p).

||Scoring was calculated by awarding one point for adherence to open-ended (no intervals) recommendation sub-groups in NNR; physical activity (1p), fiber intake (1p), sodium intake (1p), alcohol intake (1p), mineral and vitamin intake (1p), and one point to each sub-group in macronutrient recommendations; sugar intake (1p), saturated fat intake, essential fat intake (1p), and protein intake (1p).

Using the same scoring criteria as the initial model, we excluded those with daily or weekly multivitamin use (about 12 % of all participants), but the results were similar to the initial model including all multivitamin supplement users (IRR 0.93, 95% CI 0.76-1.15) for high adherence to the NNR (>5.5 points) compared with low adherence (<4.5 points).

We found no association between URTI and adherence for any individual group, except for physical activity, which was associated with a reduced risk of URTI (IRR 0.82, 95% CI 0.69-0.97). Single vitamins and minerals were not included in the analysis due to the large number of individual vitamins and minerals.

To study the possible continuous relationship between adherence to the NNR and URTI risk, we fit spline regression for the initial scoring model. We also fit spline regressions for each group of individual NNR recommendations and URTI risk. Only physical activity appeared associated with URTI risk (Figure 17).

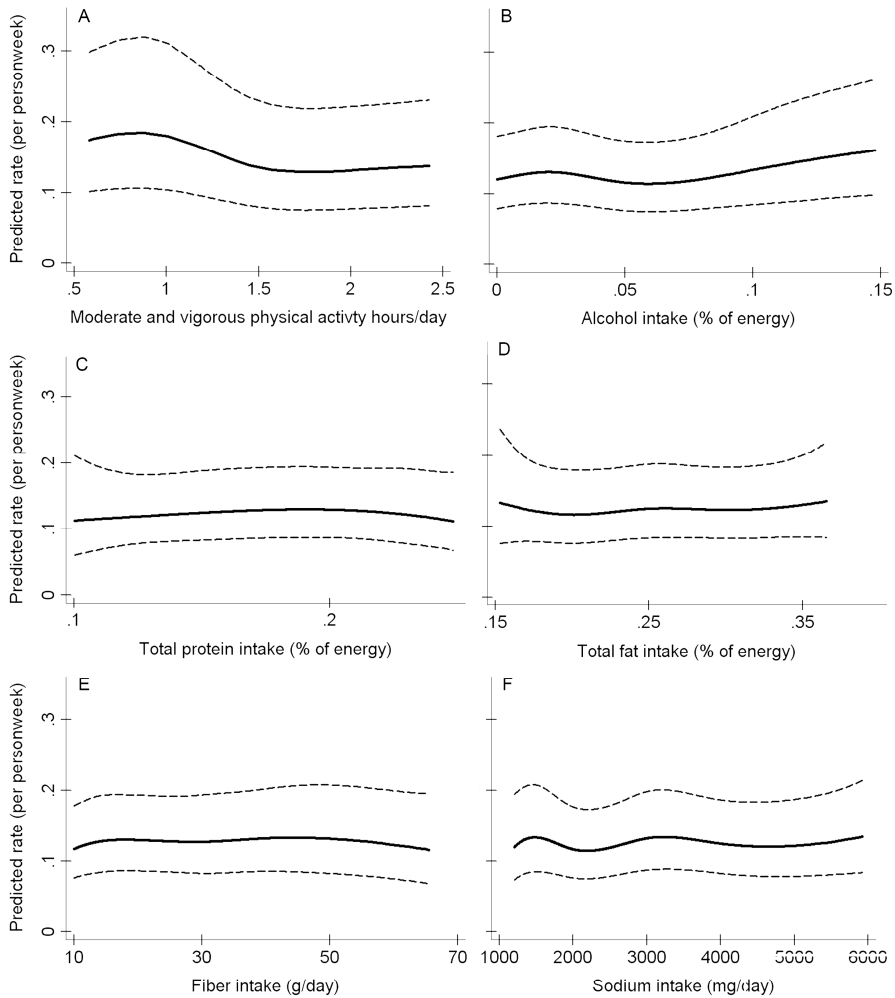


Figure 17. Smoothed incidence rates of URTI for groups of individual recommendations in the Nordic Nutrition Recommendations (NRR) using absolute intake or hours of physical activity (122).

7.5 LIFESTYLE FACTORS AND IMMUNE FUNCTION

In paper V, mean sleep duration on the night before blood draw (prior sleep) was 6.9 hours (range 4-10), whereas mean time for sleep/rest on a typical weekday (general sleep/rest) was 7.7 hours (range 6-10).

From analyses of repeated measures on the same individual, adjusted for time awake before blood draw, short prior sleep (<7h) was found to be associated with a higher PHA-stimulated T cell function (factor 1.49 [95% CI 1.07; 2.09]) compared with normal prior sleep (7-9h) (table 10). In contrast, NKCA was lower for short prior sleep (<7h) compared with normal prior sleep (7-9h) (factor 0.70 [95% CI 0.54-0.92]) (table 10). However, no notable differences were found between short prior sleep and normal prior sleep on immune cell counts or B cell function (table 10). The differences among short and normal prior sleep on T cell function and NKCA are also presented graphically by adjusted means and corresponding 95% CIs in figure 18.

Analyses on general sleep/rest duration (during a typical 24 hour period) using between-subjects analyses showed no clear association of short general sleep/rest (<7.5h) and immune cell counts, T cell function, NKCA, or B cell function compared with a general sleep/rest of 7.5-9.5h.

High levels of general physical activity (≥ 38 MET-hours/day on a typical 24 hour period) were associated with a higher number of circulating lymphocytes, in particular T cells (factor 1.28 [95% CI 1.02; 1.61]) and B cells (factor 1.47 [95% CI 1.17; 1.85]), but not NK cells (factor 0.98 [95% CI 0.64; 1.50]) when comparing with low general physical activity (<38 MET-hours/day). In addition, we found that high general physical activity was potentially associated with a higher NKCA and higher B cell function compared with low general physical activity (factor 1.56 [95% CI 0.91-2.66]) and (factor 1.46 [95% CI 0.93-2.28]), respectively, although the results are inconclusive as the CIs are wide. General physical activity was not greatly associated with T cell function in our study.

High perceived general stress (score ≥ 21) compared with low perceived general stress (score <21) was associated with a higher T cell function in response to PHA (factor 1.39 [95% CI 1.00; 1.94]). However, perceived general stress was not greatly associated with immune cell counts, NKCA, or B cell function.

Results from prior sleep and perceived stress show similar increases in T cell responses to PHA. In order to separate the effects of prior sleep and perceived stress, we excluded those with less than 7 hours of sleep on the night prior to blood draw when analyzing perceived stress, but there was no considerable change in estimate. Similarly, we excluded those with high perceived stress (score ≥ 21) when looking at prior sleep, but again results remain similar to main analysis.

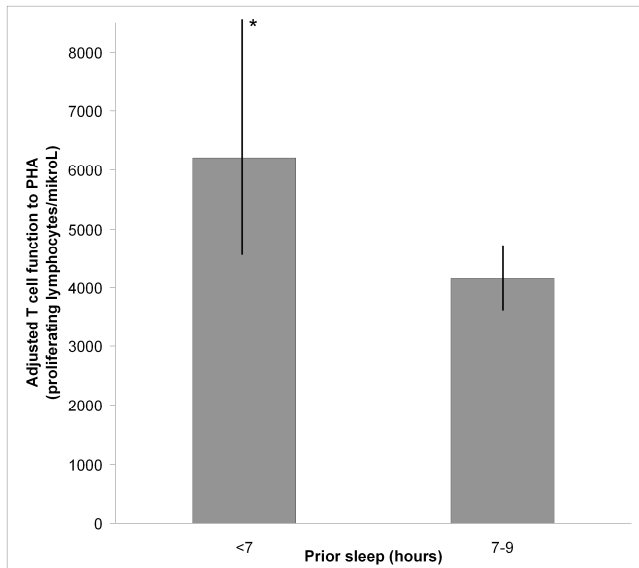
Within-subject analyses were also made of cortisol and immune cell counts and immune function, but a high cortisol concentration (>350 nmol/L, median cut-off) was not associated with NKCA, T cell function in response to PHA, nor greatly associated with any other measure of immune function or immune cell count.

Table 10. Regression statistics for within-subject analyses of prior sleep on night before blood draw and immune cell counts and function. Normal prior sleep (7-9h) was used as reference group for all analyses.

	No. of participants	No. of measurements	Adjusted for time awake before blood draw	
			Factor ^a	95 % Confidence Interval
Lymphocytes (cells/ μ l)	29	83	1.09	0.91-1.29
T cells (cells/ μ l)	29	83	1.09	0.91-1.31
NK cells (cells/ μ l)	29	83	1.08	0.77-1.51
B cells (cells/ μ l)	29	83	1.10	0.93-1.31
T cell function measured by proliferative responses to (cells/ μ l):				
mitogen PHA	19	56	1.49	1.07-2.09
Superantigen	18	50	1.30	0.54-3.12
SEA+B				
specific antigen	19	56	1.34	0.58-3.11
Influenza				
NK cell activity (NKCA) measured by cytotoxic responses to K562 cells	13	39	0.70	0.54-0.92
B cell function measured by proliferative responses to mitogen PWM	19	56	0.90	0.53-1.51

^aThe exponentiated coefficients and 95 % confidence intervals estimate the multiplicative change in the outcome variable when the exposure is increased by one unit, i.e., comparing short prior sleep with normal prior sleep.

Prior sleep and T cell function to PHA



Prior sleep and NKCA

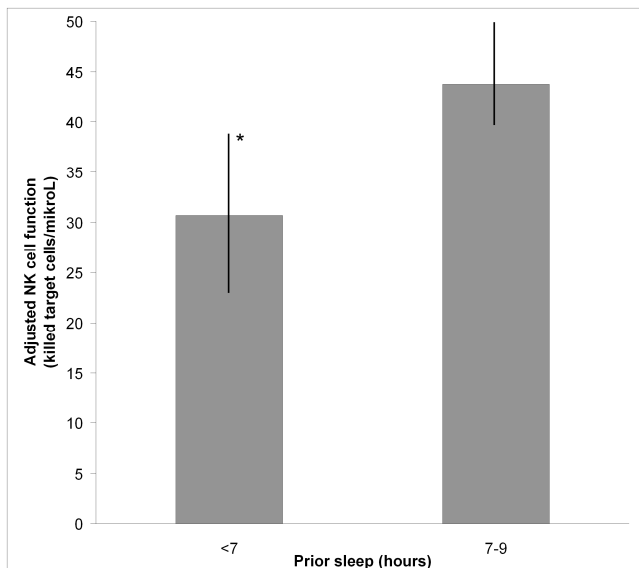


Figure 18. Prior sleep and immune cell function using within-subject design and adjusted for time awake before blood draw. Error bars are 95% confidence intervals. * p-values are 0.02 and 0.01, respectively.

8 DISCUSSION

In this section, the underlying methodology will be explained and potential sources of error will be discussed. Thereafter a discussion of the findings and implications of each of the papers will follow.

8.1 METHODOLOGICAL CONSIDERATIONS

8.1.1 Study Design

The LIME study is a prospective cohort study studying incidence of URTI where the exposure is assessed before the outcome. Since URTI is a frequent disease, frequent follow-ups are needed which can be expensive and time-consuming if traditional paper questionnaires are used. Therefore, Web-based questionnaires may be a more suitable method.

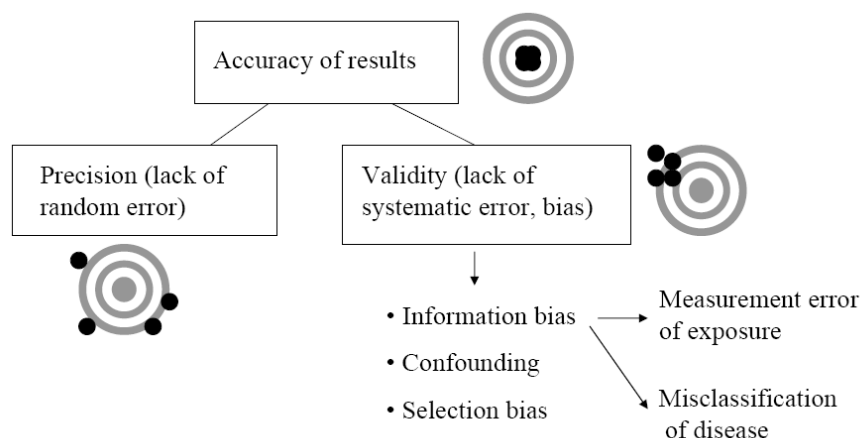


Figure 19. Precision and validity in epidemiological studies.

8.1.2 Precision and Validity

In epidemiological studies, the accuracy of an effect estimate is dependent on both systematic and random errors. Validity is lack of systematic error and precision is lack of random error. Random error is often manifested as a wide CI, but can be reduced by using a larger sample size (118).

8.1.3 Selection Bias

If the association between exposure and outcome is different among those participating in a study compared with those eligible to participate, then there is potential selection bias (118). In order to participate in the LIME study (Papers II-IV), access to a computer, Internet and an e-mail address was required. It could be argued that the LIME study is therefore not entirely population-based, and that frequent Internet users, health-conscious, and physically active individuals might be overrepresented in the study sample. This might have led to a limited range of exposure that does not reflect that of the invited study sample, which might have prevented us to find potential associations. However, the LIME study reflects well the general Swedish population

with regards to a number of variables, including the prevalence of asthma, smoking, and obesity, aside from the women in the LIME study who were slightly less obese than women in the general Swedish population (123, 124). Although exposure range may differ between those that participated and those that did not participate, it is unlikely that the association between exposure and outcome would differ, which is a criterion for selection bias.

8.1.4 Measurement Error of Exposure

Measurement error of exposure is a form of information bias and can be non-differential or differential. The measurement error is differential when the exposure errors are related to the outcome, which then can either over- or underestimate the true association. Measurement error of exposure can be a concern when exposure is self-reported. Memory, education, unwillingness to report an unhealthy lifestyle, and poor self-knowledge might influence the accuracy of the information given. If the measurement error of exposure is unrelated to the outcome (non-differential), then the true association is likely to be diluted if the exposure is dichotomous, but can under some circumstances lead to bias away from a null effect.

Categorization of exposure can also lead to measurement error of exposure. Effect estimates often change depending on where cut-offs are placed. Biological or pre-set cut-offs are preferred, for example BMI. The median can be used as a cut-off and thus getting two equally sized groups, and quantile cut-offs are often used. However, the use of quantile cut-offs could lead to cut-off intervals that are too close together, with no meaningful differences in exposure between the groups. For example, in an exposure range of 0-100 with tertile cut-offs at <40, 40-42, and >42, the exposure in the lowest and the highest groups are very close to each other and differences in outcome between the groups are likely very low. Instead, by using arbitrary cut-offs, for example <35, 35-45, >45 there is a greater range in exposure between the highest and the lowest exposure group (118). By keeping an exposure variable continuous, for example by using spline regression, the measurement error of categorization can be avoided.

8.1.4.1 Physical activity assessment

In Paper II, those with low education reported fewer URTI, and a previous validation study of the physical activity questionnaire reveal that those with low education overreport their physical activity levels (107). Because of this, there may be differential measurement error of exposure. To investigate this, we excluded those with education at a lower level than college (33% of participants excluded). Results remained similar to main findings with a reduced URTI risk of 15% compared with 18% in the main analysis. This indicates that any potential differential measurement error of physical activity by level of education was limited, and did not account for the overall result.

8.1.4.2 Food frequency questionnaire

FFQ is a commonly used method to assess diet in epidemiological studies. It is easy to administer and the burden for the participant is relatively low. Also, in most epidemiological studies, the aim is to rank individuals according to their intake, rather than to assess the absolute intake. However, there are limitations. The FFQ does not assess the entire diet, but large parts of the dietary intake. The shortcomings of a FFQ

can blunt a true association (125), and therefore results based on a FFQ may be interpreted with caution. For example, a European study (EPIC) using both FFQ and food diaries, found a stronger association between saturated fat and breast cancer based on data from food diaries compared to FFQ (126). In dietary surveys, regardless of dietary assessment method, fat, especially saturated fat, and carbohydrates are considered to be typically under-reported, while protein is typically over-reported (127). Obese and weight-conscious participants are considered to be those who under-report unhealthy food the most (128-130). A previous validation study using the same FFQ and repeated records of the total dietary intake, found that the correlation coefficients between FFQ and dietary records were moderate to high for different nutrients (ranging 0.31 for iron to 0.81 for vitamin C). The intake of various nutrients in Papers I-IV were in the same magnitude as the intake reported in a previous national Swedish study, Riksmaten, from 1997-1998 of 1215 Swedish men and women (131). In Riksmaten, the participants were asked to record their total intake of food and beverages using a seven-day food diary, and did not use a FFQ.

Moderate validity for certain food items or nutrients may result in bias, and double-ended macronutrient recommendations (example: 50-60 % of energy from carbohydrates) are more vulnerable to poor validity (i.e. misclassification when using FFQ) than open-ended recommendations (example ≤ 10 % of energy from saturated fats). In alternative scoring model 3, we excluded all double-ended recommendations, but the results did not change much compared to the initial scoring model. In order to reduce bias from measurement error using absolute cut-points for the NNR-scoring, we used a continuous score proportional to the distance from the guidelines (in all scoring models). By doing this, intakes close to, but not within the recommended intake were still given high points in the scoring system.

8.1.4.3 Sleep

Sleep was self-reported in the LIM study, which could lead to non-differential bias that is likely towards a null effect, since self-reporting of exposure is unlikely to be associated with the outcome in the LIM study (Paper V).

8.1.5 Misclassification of Disease

Misclassification of disease may be a concern when disease is self-reported. If disease classification is different between exposure groups, then the misclassification is differential. Self-diagnosis of URTI has been shown to be reliable in adult patients (132). However, Spence et al. (133) showed that not all self-reported URTI have a verified pathogenic cause, making it difficult to distinguish between symptoms and infection. Since we studied self-reported URTI symptoms and not verified infections, misclassification of disease is possible in Papers II-IV, but it is unlikely that this has affected the result greatly after controlling for both pollen allergy and smoking, factors known to be associated with URTI-like symptoms. Furthermore, we adjusted for potential confounding factors in the final statistical models, but unmeasured aspects of a healthy lifestyle might cause bias.

8.1.6 Confounding

When studying associations in epidemiological studies, it is difficult to attribute an observed association to a single exposure. There is always the possibility of a mixing of effects. A confounder is a factor that is associated with both the exposure and the outcome, and can either falsely show an association between exposure and outcome, or

mask such an association. Figure 20 illustrates an example of confounding: Vitamin C is associated with a low risk of URTI. However, some of this effect appears to be confounded by age, since high age is associated with low URTI and a high intake of vitamin C. After adjusting for age, the association between vitamin C and URTI is likely to become weaker.

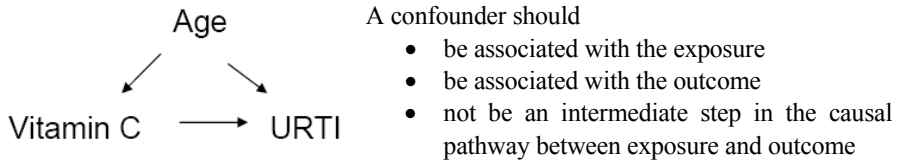


Figure 20. An example of confounding.

Confounding can be controlled for in the statistical analyses by stratifying or regression analyses. Confounding can also be dealt with in the design. For example, by only including women in a study, confounding by sex is prevented. In Paper II, III, and IV confounding was dealt with in the multivariable regression model. In Paper V, the study population was small, making adjustments for confounding factors difficult in analyses on general sleep, general physical activity, and perceived general stress without yielding uninformatively wide CIs. However, by using a within-subject design for analyses on prior sleep, the need to control for confounding was limited. Adjustments were however made for number of hours awake before blood draw, since that varied from day to day.

8.1.7 Residual Confounding

Unmeasured potential confounders, such as hand washing, hygiene, or other unmeasured aspects of a healthy diet or lifestyle may have introduced confounding that we could not control for. For example, in Paper III, it is possible that unmeasured phytochemicals such as polyphenols found in vitamin C-rich plant foods may have confounded the association between dietary vitamin C and URTI. It is also possible that other unmeasured sources of vitamin C and vitamin E, including herbal products and food additives, may have affected the association, but that type of confounding would likely have led to an underestimation of a potential association.

In addition, categorization of continuous confounding factors, for example age, may be a reason for residual confounding. When controlling for age in Papers II-IV, age was categorized in 10-year-intervals. In order to see if there was residual confounding by age, 5-year-intervals were used in additional analyses on physical activity and URTI, but the main results did not change.

8.1.8 External Validity

External validity, or generalizability, is making predictions about other populations based on results from the present study population. If internal validity is high, the findings are likely to be valid in other populations as well. The LIME study is a population-based study, but study participants may be more likely to use the Internet and be more health conscious compared with non-responders. However, since

responders and non-responders alike did not know at baseline if they were going to contract a URTI during the course of the study, it is unlikely that non-responders systematically would have changed the results if they had participated.

8.1.9 Reverse Causality

Because empirical science cannot provide any logical proofs, causality can never be proven in any type of study, even the best-designed experiments. Reverse causality can occur when a putative outcome is actually a predictor for the putative exposure, rather than the reverse. For example, one may theorize that inactivity causes obesity, but it may be that obesity causes inactivity, leading to an association that is causal in the reverse of the hypothesized direction. In the LIME and LIM studies, reverse causality is an implausible explanation for the results because participants with a URTI at baseline were excluded from analyses.

8.2 GENERAL DISCUSSION OF FINDINGS AND IMPLICATIONS

8.2.1 Feasibility of Web Questionnaires

Results from the FAS study (Paper I) show that Web questionnaires can be used for research purposes in population-based settings where the Internet access is high, although the initial response rate was lower than for the traditional printed questionnaire. By comparison, the willingness to answer a second questionnaire was higher when using a Web questionnaire instead of a printed questionnaire, which suggests that those that responded to the Web questionnaire found the process more appealing than those who responded to the mailed questionnaire. Personalized feedback in the Web questionnaire further increased the compliance rate for a second questionnaire. Total response rates for the second part of the questionnaire were similar for the printed and the Web questionnaires.

The initial response rate for the Web questionnaire in LIME was low (36%), which was expected since participants were only given three weeks to enter the study. In contrast, the continuation rate (compliance) for each follow-up was high, i.e. more than 80% of the participants responded to each follow-up Web-questionnaire. This further supports the use of Web questionnaires when multiple follow-ups are needed.

The area in Sweden where the FAS and LIME studies were performed has one of the highest percentage of fast Internet connections in the world due to the fact that the county installed fiber optic cables to private households in the 1990's. 95% of the households in both urban and rural areas were connected to the Internet in 2009 (134).

The largest cost in epidemiological research is the handling of paper questionnaires (printing, postage, scanning, and phone interviews to correct incomplete or implausible answers). Since the start of the FAS and the LIME studies, the use of Web questionnaires has become a standard method for collecting data at the Department of Medical Epidemiology and Biostatistics at Karolinska Institutet, and other places.

8.2.2 Physical Activity, Stress and URTI

Our results confirm the findings from other studies suggesting an inverse association between moderate to high levels of URTI (27, 30, 135, 136). However, we did not observe a J-shaped association between physical activity and URTI risk, as suggested by Nieman (38). This discrepancy might be explained by the paucity of participants in our study whose physical activity level corresponded to neither the intensity nor duration to that of a marathon runner, for whom increased risk of URTI has previously been observed (38). A participant classified as having a high physical activity (>55 MET-hours / day) in our study would for example be someone with a sedentary job that goes jogging or to the gym for an hour each day and is moderately active the rest of the day (for example takes the stairs, does household chores, or actively plays with the children).

A previous epidemiological study (48) as well as experimental studies (see (49) for a review) have shown a progressively increasing risk of URTI with increasing levels of psychological stress. A cohort study among 1149 university employees reported a 2.5-fold increased risk of common cold among participants reporting high perceived stress levels (48). It has also been shown that a high level of perceived stress is associated with increased symptoms of illness and increased production of Interleukin-6 in response to a viral challenge (137). We used the 14 items validated version of the PSS by Cohen (41) to assess perceived stress during the previous month, but we did not find an overall effect of high perceived stress on URTI compared with low stress. Nor did results change when we only included the first three follow-ups in order to minimize the effect of potential change in perceived stress occurring during the course of the study. However, it is possible that perceived stress assessed in this population-based study was not severe, or long-term, enough to affect immune function. We saw an interaction between stress and physical activity on immune function, and this interaction has previously been observed in animal studies, suggesting that regular, moderate physical activity reduces the negative effects of an acute stressor on the immune response (for a review, see Greenwood and Fleshner (138)).

We observed a difference between men and women regarding the association between perceived stress levels and physical activity. This difference might possibly be explained by a proposed difference in physiological and behavioral responses to stress for men and women (55). The “fight-or-flight” response to stress, while present in both men and women, is proposed to be stronger in men, and a “tend-and-befriend” response more common in women in response to stress (55). These response differences could explain why men might benefit more from physical activity while under stress than women.

We saw a decline in URTI with increasing age, which is in line with previous studies (20). As we get older, we are more likely to encounter viruses that our immune system recognizes, which may explain part of this decline. On the other hand, there is a documented overall decline in immune function among elderly (age >65) (see Panda et al. for a review (96)), but the participants in our study were most likely too young (age 20-60) for this decline to be observed.

Previous studies have found that physical activity is associated with lower influenza-related mortality (139). However, when we separated URTI into systemic URTI including fever (sign of influenza) and non-systemic URTI without fever (indicates common cold) in an attempt to differentiate between common cold and influenza, we

found no protective associations of physical activity on systemic URTI, but these results are based on a limited number of participants. Also, participants in our study were not specifically asked to check their temperature and therefore we might have over or underestimated the number of cases with fever.

Our findings confirm that high physical activity is associated with a reduced risk of URTI, and indicate that the effect of physical activity in preventing URTI is greater among highly stressed people, especially among men, which is a novel finding. These findings may have important implications to populations with a high incidence of URTI.

8.2.3 Vitamin C, Vitamin E, and URTI

We observed a reduced risk of URTI for a high vitamin C intake from food among women, but not among men and a possible explanation may be that the vitamin C intake from food was higher among women than among men. The difference in vitamin C intake from food might also explain why we saw a possible protective effect of vitamin C supplement use among men, but not among women, since the high intake from food could make supplement use redundant in women.

A previous epidemiological study on combined vitamin C intake from food and supplement use did not find a reduced risk of URTI. Estimates were IRR 1.1, 95% CI 0.8-1.4 for men and IRR 1.0, 95% CI 0.7-1.3 for women for the highest quartile intake group (median 256.5 mg/day for men, 333.5 mg/day for women) compared with the lowest quartile intake group (median 40.3 mg/day for men, 57.7 mg/day for women) (71). The investigators did not separate vitamin C intake from food from that of supplement use. Also, they used a limited FFQ that only assessed vitamin C and zinc intake, making adjustments for other nutrients impossible.

A recent review of over 30 trials studying the effects of vitamin C supplementation on common cold prevention reports that vitamin C supplementation is not associated with fewer colds (risk ratio 0.96, 95% CI 0.92-1.00), but is associated with a modest 8% reduction in the duration of the common cold (95% CI 3% to 13%). The same review also reported a reduction in incidence only for physically active individuals, for whom the risk ratio was 0.50 (95% CI 0.38, 0.66) (66). We did not find any large difference in protective effect of vitamin C from food on URTI among highly physically active compared with those less physically active.

A high vitamin E intake from food was not associated with a lower URTI risk in our study, and comparative studies are lacking. It is possible that the range of vitamin E intake was too narrow (2-20 mg/day) to detect a potential effect on URTI.

We found a protective effect of daily or weekly vitamin E supplement use on URTI risk among men, but not among women. Previous studies on vitamin E supplementation report conflicting results and have focused on the elderly (69, 70). A large intervention study of vitamin E and beta-carotene supplements in smoking Finnish men found no association between dietary intake of vitamins C, E, or beta-carotene and URTI risk, nor did they see protective associations for vitamin C supplement use by own initiative (140). However, they found that vitamin E supplement use was associated with a lower risk of URTI among all smoking men (140), but the effect among men over the age of 72 was divergent: a lower risk of URTI was seen among city-dwelling, light smokers (5-14 cigarettes /day) compared with control group, but intriguingly an increased risk

of URTI (RR 1.58) was found among city or country-dwelling heavy smokers (>15 cigarettes / day) (141). We found no indication of any important effect measure modification between age and dietary or supplementary vitamin E or C intake on URTI risk. Neither did we find any important effect measure modification between smoking and dietary or supplementary vitamin E or C intake on URTI risk. It is possible that participants in our study were not old enough, or did not smoke enough for any effect measure modification to be evident. Another trial, among the elderly, found that the plasma concentrations of vitamin E supplements may be decreased by a concomitant intake of fish oil supplements (142). In our study, few participants took both fish oil supplements and vitamin E supplements regularly, and we did not find any large effect measure modification on dietary or supplementary vitamin E intake by fatty fish intake.

Observed intakes of vitamin E were below the recommended intake levels for most participants (recommended intake of vitamin E is 10 mg/day in Sweden and 15 mg/day in the US), which has also been observed in previous studies, including a large American study (CSFII) (143) where diet was measured using 24 hour recalls, and a Swedish study, Riksmaten, using a 7-days food diary (144). It is possible that the intake of vitamin E from food in our study population was too low to produce a protective effect on risk of URTI.

Possible benefits of vitamin E supplementation should be weighed against possible adverse effects, inasmuch as vitamin E supplementation has been reported to be associated with a small increase in total mortality (RR 1.04, 95% CI 1.01-1.07), mainly from cancer and cardiovascular disease (see (145) for a review). The same review found no increased risk for death associated with vitamin C use. Antioxidants are considered instrumental in protecting cells from oxidative agents and free radicals. Nevertheless, it has also been suggested that large doses of antioxidants from supplements can limit the own body's defense system. Free radicals cause cell damage, but they also trigger the immune system to induce apoptosis. Therefore large doses of supplements may possibly prevent natural defense mechanisms of the immune system (145).

The primary clinical and public health implication of our finding is that intake of vitamin C from food might suffice to lower the risk of URTI among women. In addition, supplement use of vitamin E and possibly vitamin C may reduce risk of URTI among men.

8.2.4 Nordic Nutrition Recommendations and URTI

To the best of our knowledge, there are no other studies available that have studied the effect of dietary recommendations on the risk of URTI. Previous studies evaluating dietary recommendations have focused on the effect on diseases such as myocardial infarction or cancer. McCullough et al. (76, 77) evaluated the adherence to the DGA using ten equally weighted groups (e.g. servings of grain, fruit, and meat). They found no preventive effect for cancer and no effect on cardiovascular diseases in women (77), and only a small reduced risk for men (76). A more recent study, using a continuous scoring model similar to ours, found an association between DGA and atherosclerosis progression in women (80). It is possible that the use of food group recommendations based on such as DGA is more efficient in predicting disease than the NNR which emphasize nutrients.

We found that the overall adherence to the NNR was moderately good. Overall adherence to the NNR was not associated with URTI risk in our study. However, when investigating individual components of the NNR, we found that high physical activity was associated with a lower URTI risk. Results from this study suggest that the NNR is not a good tool to prevent URTI.

We cannot rule out the possibility that the null results for overall adherence to the NNR in this study is due to participants being generally very well-nourished, leading to a limited variation and skewing towards higher adherence scores for certain recommendations in order to see an association with URTI. Nonetheless, small variations in the scoring was taken into account by using a continuous grading scale for intakes close to NNR cut-offs. Further analyses of single vitamin and mineral intakes and food groups may provide more insight on the relevance of NNR and URTI.

8.2.5 Lifestyle Factors and Immune Function

Paper V demonstrates that naturally occurring short prior sleep on the night before blood draw (less than 7h) is related to higher T cell function in response to PHA as well as lower NKCA in morning samples compared with normal prior sleep (7-9h). These findings support an earlier study of partial sleep deprivation during 5 days showing increasing T cell function (92). Other studies show decreasing T cell function following severe sleep deprivation (24 - 48 h) (91, 146), indicating that T cell function may decrease or increase depending on the degree of sleep deprivation. While the present study as well as the studies by Boyum et al. (92) and Irwin et al. (89, 90) have studied the effects of rather moderate sleep deprivation, most previous studies have focused on more severe sleep deprivation (see review (17)). The implication of the higher T cell function observed for short sleep is not clear, but we speculate that it might be part of the development of diseases involving overactive T cells, for example autoimmune diseases. It has recently been highlighted that disturbed sleep is common in several autoimmune conditions characterized by overactive T cell function (147). A possible mechanism for this is through regulatory T cells, which are important for suppressing inflammatory responses and achieving immune homeostasis (148). Experimental sleep deprivation in healthy individuals has been shown to disturb the function of regulatory T cells (149), a dysfunction which is related to both T cell proliferation (150), and also common in patients with autoimmune diseases (see (151) for a review).

This study also supports earlier experimental studies showing that acute sleep restriction is related to lower NKCA (89, 90). Previous observational studies on natural sleep have found a lower NKCA among depressed, in elderly with poor sleep, and in shift-workers (see (152) for a review). In studies on healthy volunteers, no association was observed between natural sleep duration and NKCA between subjects (95, 153), but when a within-subject design was used, lower NKCA was associated with short sleep duration for repeated measurements on the same women (153). In addition, our results are in line with a recent prospective study on natural sleep showing that self-reported sleep duration of less than 7h increased the risk of infection 2.94 times (95 % CI 1.18-7.30) after an experimentally induced viral challenge in 153 healthy men and women aged 21-55 (16).

There were no strong associations between general short sleep/rest (<7.5 hours) and any measured immune parameter, suggesting that the acute effects are greater and may mask possible long-term effects, at least in this study population. Another explanation may be that the question of general sleep duration was not sufficiently well-defined, as

it also included time at rest in its measure as well. It is also likely that rating the sleep duration for prior sleep on night before blood draw is a more reliable measure than rating general sleep/rest.

The present study controlled for the possible impact of prior physical activity (no strenuous activity was allowed 24 hours prior to blood sampling). With respect to general physical activity, it was shown that participants with a higher level of general physical activity had higher circulating numbers of T cells and B cells and a potentially higher B cell function and NKCA. Comparable studies of B cell function and physical activity are lacking to our knowledge, but some studies suggest that NKCA may be slightly elevated among rested athletes compared to sedentary controls (9, 36, 37). Implications of findings regarding cell numbers are however limited, since changes are likely to be due to redistribution of cells rather than apoptosis. Despite controlling for activity prior 24h, it cannot be excluded that strenuous physical activity earlier than 24h may have affected the results.

In our study, we found an association between high perceived general stress and a higher T cell function in response to PHA. Previous studies have found that acute stress upregulates innate immunity, and downregulates adaptive immunity, while chronic stress suppresses most immune responses including NKCA and T cell function in response to PHA (see (10) for a review). In the same review it was found that subjective stress, which may be comparable to perceived stress in our study, was not generally associated with immune function. It is possible that the perceived stress assessed in our study was not severe or long-term enough to show the suppression observed for chronic stress. In addition, it is likely that the method used to measure T cell function in our study, FASCIA, was able to capture associations not previously observed since it has been shown to be more accurate and informative about immune status than conventional methods measuring cell mediated immunity (104).

There are several limitations with the present study, including the study of multiple exposures and immune parameters, which increases the risk of false positives. Strengths in our study include the use of repeated measures on the same individual for analyses of prior sleep. This within-subject approach reduces the impact from between-subjects variations, a particular concern when studying immune function that may vary greatly between individuals.

This study sheds light on possible mechanisms involved in the increased disease risk that is caused by short sleep duration. More specifically we here show that natural occurrences of short sleep were associated with lower NKCA which might impair ability to fight infections. The implication of the higher T cell function observed for short sleep and high perceived stress is not clear, but we speculate that it may be involved in development of diseases involving overactive T cells, for example autoimmune diseases. These findings may have important implications to populations with increasing prevalence of impaired sleep.

9 CONCLUSIONS

In this thesis, we have studied the use of Web questionnaires and potential associations between lifestyle factors and immune function. We found that:

Paper I:

- Web questionnaires can be useful tools for collecting research data in populations with a high percentage of Internet access.
- Interactivity in the Web questionnaire increased response rate for a follow-up questionnaire compared with a printed questionnaire or a Web questionnaire without interactivity.

Paper II:

- High physical activity was associated with a lower risk of URTI.
- Highly stressed people, particularly men, seemed to benefit more from physical activity than those with low stress levels.

Paper III:

- High vitamin C intake from food was associated with a lower risk of URTI among women, but not among men.
- Supplement use of vitamin E, and possibly vitamin C, may be preventive of URTI among men, but not among women.

Paper IV:

- Overall adherence to the Nordic Nutrition Recommendations was moderately good.
- Overall adherence to the Nordic Nutrition Recommendations was not associated with URTI risk.
- The individual recommendation in the Nordic Nutrition Recommendations regarding physical activity was associated with a lower risk of URTI, but none of the individual dietary recommendations were associated with risk of URTI.

Paper V:

- Short sleep duration on the night before blood draw was associated with lower NK cell activity and higher T cell function.
- High perceived stress during the last month was associated with a higher T cell function.
- High general levels of physical activity was not associated with immune function, but was associated with a higher circulating number of B cells and T cells.

10 SAMMANFATTNING PÅ SVENSKA (SUMMARY IN SWEDISH)

Vårt immunförsvar är komplicerat. Många av dess funktioner och hur det regleras är okända än idag. Vi har studerat förekomst av självrapporterade övre luftvägsinfektioner som en markör för immunförsvarets förmåga att stå emot infektioner. I övre luftvägsinfektioner ingår förkylning och influensa. Kostnaden för enbart förkylningar beräknas vara 40 miljarder dollar årligen i USA och luftvägsinfektioner är i många länder den vanligaste anledningen till varför man söker vård. Trots detta finns det lite kännedom om hur man kan minska mottagligheten för infektioner. Livsstilsfaktorer som fysisk aktivitet, stress, sömn och kost påverkar immunförsvaret och i denna avhandling undersöktes om och hur Internet kan användas för att samla in data samt om det finns samband mellan livsstilsfaktorer, luftvägsinfektioner och immunförsvar.

I delarbete I undersöktes möjligheten att använda webbenkäter i en populationsbaserad studie. Jämfört med traditionella pappersenkäter fann vi att webbenkäter gav lägre initial svarsfrekvens, men högre svarsfrekvens för efterföljande enkäter. Detta gällde särskilt webbenkäten med personlig återkoppling. Baserat på dessa resultat genomfördes en populationsbaserad kohort bestående av 1509 män och kvinnor i åldern 20-60 som enbart fyllde i webbenkäter. Uppföljningsperioden var fyra månader och studien ligger till grund för delarbete II-IV. Deltagarna rapporterade totalt 1181 luftvägsinfektioner. Resultat från delarbete II visar att höga nivåer av fysisk aktivitet (55 MET-timmar/dag, MET, metabolic equivalent task) var associerat med en 18% lägre risk att insjukna i luftvägsinfektioner jämfört med låga nivåer av fysisk aktivitet (<45 MET-timmar/dag). Vi fann också att personer med höga stressnivåer, särskilt män, verkade ha större skydd av fysisk aktivitet än de med lägre stressnivåer. När intaget av antioxidanter och risken för luftvägsinfektioner studerades i delarbete III, fann vi att ett högt intag av vitamin C från mat (>200 mg/d) var associerat med en 40% lägre luftvägsinfektionsrisk jämfört med ett lågt intag (<100 mg/d) bland kvinnor. Detta samband fanns inte hos män, som överlag hade ett lägre intag av C-vitamin jämfört med kvinnor. Kosten undersöktes vidare i delarbete IV, där följsamhet till de nordiska näringsrekommendationerna (NNR) undersöktes som ett mått på ett hälsosamt kostintag. I NNR ingår rekommendationer angående makronutrientier (t.ex. mättat fett), mikronutrientier (vitaminer och mineraler), fiber, salt, alkohol och fysisk aktivitet. Ett flertal modeller för poängsättning användes och samtliga modeller indikerar att god följsamhet till NNR inte påverkade risken för att insjukna i luftvägsinfektioner. I delarbete V undersöktes naturliga variationer av sömnlängd, stress och fysisk aktivitet jämfört med antalet leukocyter och dess funktion bland 36 män och kvinnor, 20-54 år gamla. Resultaten visar att kort sömn (<7 h) natten innan blodprovstagning var associerat med en 49% högre T-cellsfunktion vid stimulering med phytohemagglutinin (även kallat PHA) och en 30% lägre funktion av NK-celler jämfört med normal sömn natten innan provtagning (7-9 h). Höga stressnivåer var associerat med en 39% högre T-cellsfunktion vid PHA-stimulering jämfört med låga nivåer av stress. Resultaten visar också att hög fysisk aktivitet var associerat med en högre koncentration i blod av B-celler och T-celler, men fysisk aktivitet påverkade inte funktionen av dessa celler.

Sammanfattningsvis fann vi att Internet och web-baserade enkäter är användbara verktyg för att samla in data i forskningsstudier i en population med hög andel Internetanvändare. Vi fann även att livsstilsfaktorer är kopplat till vår förmåga att stå emot infektioner.

11 ONGOING AND FUTURE RESEARCH

The field of e-epidemiology continues to grow. Web questionnaires are currently being used in many large epidemiological studies and they are sometimes used together with traditional printed questionnaires to increase response-rate (i.e. mixed-mode). Other methods for collecting epidemiological data are being explored as well, for example the use of Short Message Service (SMS) in mobile phones, which have been used to collect information about influenza prevalence and influenza vaccination (155).

The role of physical activity in URTI prevention should be explored further through intervention studies to study if increased physical activity reduces the risk of URTI in the experiment group compared to the control group (no increase in physical activity). Resistance to URTI could be measured by recording number of URTI during the study period or by a viral challenge (e.g. viral inoculation) at the end of the intervention period.

Another intervention study of interest would be to study if high physical activity can boost immunological memory in response to immunizations compared to a control group. The aim would be to increase the effect of immunizations, since a weak immunological memory in response to immunizations is a problem in certain groups, for example the elderly (156). In the proposed intervention, it would be important to measure immunological memory prior to the immunization and physical activity intervention, since previous encounters of viruses similar to the immunization virus can affect the immunological memory.

We will use the LIME dataset to follow-up on the results from the LIM-study with regards to sleep. The aim is to study if short natural sleep and the quality of sleep are associated with the risk of URTI. Finally, in a future LIME2-study including a larger study population, it would be of interest to study if physical activity, stress, gender, and sleep interact with each other and have an impact on immune function. In the future LIME2-study, it would also be of interest to include other types of infections, such as urinary tract infections and stomach flu.

12 ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to the many people who, in different ways, have contributed to this work. In particular, I would like to thank the following:

Katarina Bälter, my principal supervisor, thank you for introducing me to the world of epidemiology and for entrusting me to work on your studies. You have a great knowledge of epidemiology. Thank you for being my teacher, mentor and friend. Thank you for always listening to my ideas and for believing in me. You give criticism in a very positive way and you have thoughtfully pushed me forward and created invaluable opportunities for me to grow academically.

Kenneth Rothman, my co-supervisor. It has been a great honor working with you. Your knowledge of epidemiology has been invaluable to the manuscripts in this thesis. Thank you for always taking the time to carefully review my work and explain epidemiological concepts. Thank you for challenging me to learn more and go further in my research and for teaching me to keep research presentations simple and elegant.

Hans Gaines, my co-supervisor. Thank you for guiding me through the field of immunology and immunological method. For entrusting me to work in the lab and explaining the methods.

Carl-Johan Sundberg, my co-supervisor, for sharing your knowledge of research methods and physiology. For giving energy boosts and pep talks. I am amazed at your ability to solve problems on the spot.

Mats Lekander, co-author on papers II and V. Thank you for introducing me to the field of psychoneuroimmunology. Your excitement for research is inspiring.

Ylva Trolle Lagerros, for your contagious energy and enthusiasm. For your many wise words, for always offering to help, and for being a friend.

Olle Bälter, co-author on papers I-IV. Thank you for your guiding me into the world of Web-questionnaires, I appreciate your knowledge and sense of humor.

Kristina Franck and *Fredrik Atterfelt*, laborators at SIIDC, for patiently showing me how to do the lab work and explaining the methods.

Anna Johansson for your tireless and extensive guidance in biostatistics, SAS programming, STATA programming, and data management.

Alexander Ploner for your statistical guidance and great explanations.

John Axelsson, co-author on paper V, for you expertise in sleep research.

Hans-Olov Adami, for making MEB a place for top quality research while keeping a friendly and open atmosphere, and for welcoming me at Harvard School of Public

Elinor Fondell

Health. *Nancy Pedersen and Henrik Grönberg* for continuing to create an inspiring research environment at MEB.

Sara Christensen, for friendship and co-authorship. It was a pleasure working with you on paper IV, you are a good writer and very thorough.

Ann-Britt Holmgren, Karin Dellenvall, Milka Krestelica, Mattias Hammarström, Ann-Sofie Andersson, Britt-Marie Hune, and Vladimir Boskov for assisting with blood draw for the LIM study.

Lena Andersson, Shanaz Mahdaviifar, Petra Jones, and Teghesti Tecleab at SIIDC for performing the immune cell analyses.

Gabriella Malmsten for invaluable assistance with my defense application.

Present and previous PhD-students at MEB, in particular *Elisabeth Möller, Linda Lindström, Christina Persson, Alexandra Ekman, Christin Bexelius, Gudrun Jonasdottir, Junmei Miao Jonasson, Anna Bergström, Maria Sandberg, Sara Öberg, Fang Fang, Fatima Azerkan, Sanna Tiikkaja, Pia Fernberg, Ninoa Malki, Ulrika Eriksson, Chantal Orgeas, Chuen Seng Tan, Anna Berglund, Therese Moberg, and Thomas Frisell* for friendship and interesting discussions.

Current and previous MEB coworkers, in particular *late Endre Kobold, Kerstin Linderholm, Torbjörn Gustavsson, Agneta Lönn, Åsa Klint, Katarina Ekberg, Ove Strind, Sanna Green, Gunilla Sonnebring, Stefan Lönn, Jan-Eric Litton, Kamila Czene, Paul Dickman, Camilla Ahlqvist, Carin Cavalli-Björkman, Kristina Glimsjö, Monica Rundgren* for creating a friendly atmosphere at MEB.

To my co-workers at Harvard School of Public Health in Boston: *Shona Dalal, Andrea Margulis, Alison Tse, Sara Lindström, and Katja Fall* for making my time at HSPH memorable.

Osher Center for Integrative Medicine at Karolinska Institutet, thank you for generous financial support of my PhD studies.

Swedish Council for Working Life and Social research (FAS), for making my visit at HSPH possible.

To all my many bonus-siblings and to my brother-in-law, *David Fondell*, for all your support. To my cousin *Björn Brindefalk* for scientific discussions and proof-readings of my work.

My wonderful brothers and sisters: *Malin*, your discipline, hard work, and care of others inspire me to do better. *Joel*, your musical compositions are amazing. *Simon*, you are a patient teacher, thank you for teaching me about math and combustion engines... *Isabelle*, we grew up as both sisters and best friends, thank you for being an extra mother for *Emmy* when she was a baby. *Richard*, thank you for your help with *Emmy & Elise*, and *Marcel*, a long time ago, you and *Richard* joined one of my lab

classes. When I look at you today, I sometimes still see two curious little boys in oversized lab coats.

Min kära svärmor *Siv Fondell*, tack för det generösa stöd du givit till oss under åren med *Emmy*, *Elise* och *Eveline*. Tack också till min svärmors mor *Edit* för hjälp med barnen, att du närmar dig 90 kan ingen tro.

My mother *Inga Lill Siggelsten Blum*, for making me feel that I can do anything. *Robert* for your support and help with the English language.

My father *Sivert Siggelsten*, for your contagious curiosity in learning new things. *Kerstin* for your encouragement and support.

My wonderful daughters *Emmy*, *Elise*, and *Eveline*, for putting a smile on my face every day. My loving husband *Calle*, you are the love of my life, and life is complete with you at my side.

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