# Effects of exercise on fluoride metabolism: Saliva or blood as an indicative marker in sport medicine for monitoring fluoride metabolism.

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A thesis submitted in partial fulfilment of the requirements of Teesside University for the degree of Doctor of Philosophy

MAIN TEXT (Part 1)

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# Declaration

I hereby declare that the work presented in this thesis is entirely my own and that, to the best of my knowledge, has never been published or presented for the award of any other degree or diploma of the university or other institute of higher education.

# Acknowledgements

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# List of Abbreviations

| | |
|---|---|
| % | Percentage |
| µmol | Micromolar |
| µmol/l | Micromole per litre |
| 3H-TC | [3H] Tetracycline |
| ABV | Alcohol By Volume |
| ACSM | American College of Sports Medicine |
| AHA | American Heart Association |
| $Al_2O_3$ | Aluminium Oxide |
| AUC | Area Under Curve |
| CI | Confidence Interval |
| $C_{max}$ | Maximum plasma concentration |
| $CO_2$ | Carbon dioxide |
| $D_3mft$ | Decayed, Missing, Filled Teeth |
| DBS | Dried Blood Spot |
| $ddH_2O$ | Double de-ionised water |
| DT | Dry Tips |
| DUFE | Daily Urinary Fluoride Excretion |
| EDTA | Ethylenediaminetetraacetic acid |
| F or F- | Fluoride |
| F-ISE | Fluoride Ion Selective Electrode |
| FPC | Fish Protein Concentrate |
| FSA | Hydro Fluorosilicate |
| FUFE | Fractional Urinary Fluoride Excretion |
| g | Gram |
| GH | Growth Hormone |
| $H^+$ | Hydrogen ion |
| $HCO_3^-$ | Bicarbonate |
| HDL-C | High Density Lipoprotein Cholesterol |
| HF | Hydrogen fluoride |
| HMDS | Hexamethyldisilane or Bis(trimethylsilyl)amine |
| $HR_{max}$ | Maximum heart rate |
| IPAQ | International Physical Activity Questionnaire |
| IRFT | Incremental Running Field Test |
| JCUH | James Cook University Hospital |
| m | Metre |
| MAPK | Mitogen-Activated Protein Kinases |
| MET | Metabolic Equivalent |
| MFP | Monofluorophosphate |
| mg | Milligram |
| mg/l | Milligram per litre |
| ml/min | Millilitre per minute |
| mM | Millimolar |
| mmHg | millimetres of mercury |
| MMPs | Matrix Metalloproteinases |
| MRC | Medical Research Council |
| N | Normal |
| N/A | Not Available |

| | |
|---|---|
| NaF | Sodium Fluoride |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium Hydroxide |
| ng/ml | Nanogram per millilitre |
| $NH_4Cl$ | Ammonium chloride |
| NMC | Nursing and Midwifery Council |
| NRC | National Research Council |
| $^oC$ | Degrees Celsius |
| PARQ | Physical Activity Readiness Questionnaire |
| PE | Physical Education |
| ppm | Parts per million |
| PTFE | Polytetrafluoroethylene |
| rcf | Relative centrifugal force |
| RER | Respiratory Exchange Ratio |
| RPE | Rate of Perceived Exertion |
| rpm | Revolutions per minute |
| SCHER | Scientific Committee on Health and Environmental Risks |
| SD | Standard Deviation |
| SL-SM | Sublingual Submandibular Saliva |
| TDFI | Total Daily Fluoride Intake |
| THC | Delta9-tetrahydrocannabinol |
| TISAB | Total Ionic Strength Adjustment Buffer |
| $T_{max}$ | Time at which maximum plasma concentration is attained |
| TSH | Thyroid Stimulating Hormone |
| UFE | Urinary fluoride excretion |
| UMAL | U.M. Association Limited |
| $VCO_2$ | Carbon dioxide output |
| VE | Ventilation |
| $VO_2$ | Maximal oxygen uptake |
| $VO_{2\ peak}$ | Maximum rate of oxygen consumption measured during incremental exercise |
| VP | Venous Plasma |
| VT | Ventilatory Threshold |
| VT1 | Ventilatory Threshold 1 |
| VT2 | Ventilatory Threshold 2 |
| VWB | Venous Whole Blood |
| WHO | World Health Organisation |
| $W_{max}$ | Workload maximum |

## Abstract

**Background:** The preventative role of topical fluoride in dental caries has long been established. Exposures to low levels of fluoride have been shown to reduce dental caries by 50%. However, excessive exposure to systemic fluoride during enamel development can lead to development of dental fluorosis. The metabolism of fluoride may be affected by many factors including genetics, diet and exercise. However, the methods to measure fluoride absorption and fluoride excretion which provide an indication of fluoride metabolism often involve practicality and ethical issues, especially in relation to blood collection in young children.

**Aims:** This thesis aimed to assess the use of Dry Tips and Lashley cups for the collection of ductal saliva (Study 1), as well as collecting and investigating the relation between fluoride concentrations in whole blood and plasma (Study 2A) and blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva (Study 2B). The main aim of this thesis was to investigate the effect of different intensities of continuous exercise on fluoride metabolic responses in adults (Study 3). This was followed by aiming to develop a feasible and ethically sound protocol for an experimental study on the effects of exercise on fluoride excretion in children (Study 4).

**Methods:** Study 1: The endogenous fluoride contents of Dry Tips were investigated using three pre-preparation methods: 1) Direct Ashing, 2) NaOH as an ashing aid, and 3) No ashing. Lashley cups were manufactured at Teesside University and tested at three different levels: 1) Teesside University, 2) Newcastle University, and 3) Sao Paulo University. Study 2: Phase A involved the collection of 1) venous whole blood, 2) venous plasma and 3) capillary whole blood (n= 15). Phase B involved the collection of 1) whole saliva, 2) sublingual-submandibular saliva and 3) venous plasma (n= 24).

Study 3: In a 4-treatment, 4-session cross-over study, 8 healthy adults (18 - 35 years) were invited to attend the laboratory early morning in fasting conditions. Baseline urine and plasma measurements were obtained. Participants were provided with low fluoride breakfast and a 1-mg fluoride tablet. At each session, participants were asked to either rest (control) or exercise on a cycle ergometer at light, moderate and vigorous intensities (relative workload, %$VO_{2\ max}$), for 20 minutes. Blood samples were taken at 0, 30, 45, 60 and 90 minutes post fluoride ingestion. Time-controlled urine samples were also collected over 24-hours to measure the rate of UFE. Each session was separated by a wash-out

period of one week. Data were corrected for baseline and analysed using linear mixed model analysis. Study 4: An initial protocol was developed to assess the feasibility and ethical acceptance of a protocol for a prospective study on the effects of exercise on fluoride excretion in children.

**Results:** Study 1: Dry Tips contained high endogenous fluoride and challenges were faced in the collection of ductal saliva using Lashley cups. No saliva samples were collected. Study 2: A significant correlation was found between venous whole blood and venous plasma with a ratio of 0.65. Whole saliva and venous plasma had a mean ratio of 1.30. SL-SM saliva and venous plasma had a mean ratio of 0.18. Study 3: Mean±SD baseline-adjusted rate of UFE over 0-8 hours post fluoride ingestion was 73.8±96.5, 54.3±53.3, 44.1±43.4 and 50.9±74.4 µg/hour and mean±SD baseline-adjusted peak plasma fluoride concentrations ($C_{max}$) at 45 minutes post fluoride ingestion were 23.6±14.3, 102.7±58.2, 226.2±115.6 and 94.2±58.1 ng/ml for no, light, moderate and vigorous exercise, respectively. Statistically significant differences in $C_{max}$ were found between i) control and moderate exercise and ii) light and moderate exercise ($p < 0.05$). Study 4: Ethical approval was gained for a protocol designed to investigate the effects of exercise on urinary fluoride excretion in children. The pilot study was conducted with ease and great acceptability from both, the parent and child participant.

**Conclusion:** Study 1: Dry Tips are not suitable for studies measuring fluoride and Lashley cups pose practicality issues in the wider population. Study 2: Blood plasma was found to be the most suitable biomarker for fluoride absorption. Study 3: The results indicate that moderate intensity exercise might increase absorption and reduce urinary excretion of fluoride, however further understanding the mechanisms involved is required. Study 4: Although challenging, it is possible to gain ethical approval for studies investigating fluoride metabolism in children. Larger trials are required to confirm the acceptability of the protocol.

# Chapter 1 . Introduction

Fluoride, an anion of fluorine is highly abundant in the environment and only occurs as fluoride compounds that form minerals found in rocks and soil (Barbier *et al.,* 2010). The release of fluoride in the environment is highly associated with volcanic activity, weathering of rocks and industrial contaminants. The abundance of fluoride varies with geographical locations, with some areas naturally higher in fluoride in comparison to others. Although many forms are widespread throughout the biosphere, sodium fluoride (NaF) and calcium fluoride (fluorite) are the most abundant sources (World Health Organisation (WHO), 2002).

The applications of fluoride are widespread, and it primarily receives great social and political attention for its role in oral health. Although, the Scientific Committee on Health and Environmental Risks (SCHER) (2011) stated that fluoride is not essential for human growth and development, it is of nutritional and public health importance and has been regarded as the only compound for the prevention of dental caries.

## 1.1. Effects of Fluoride

The effects of fluoride on the human body are biphasic in nature. Exposures to low levels of fluoride have been shown to reduce dental caries by 50% (Welbury, 2012). On the other hand, excessive fluoride exposure can increase the risk of dental fluorosis. Increasing fluoride exposure in its different sources for the prevention of caries has led to a rise in the prevalence of dental fluorosis in both fluoridated and non-fluoridated communities (Aoba and Feierskov, 2002; Carey, 2014). This section will describe the positive and negative effects of fluoride on bone and teeth.

### 1.1.1. Bone

Fluoride displays biphasic actions on bone and Everett (2011) stated how this biphasic action has complicated the understanding of fluorides physiological effects. Fluoride can both increase and decrease bone strength. Lower concentrations induce mitogenesis whilst higher concentrations inhibit bone formation and resorption (Pak *et al.,* 1995; Golub *et al.,* 1968). Progressive accumulation of fluoride in the skeleton is associated with mineralisation

defects leading to a reduction in bone quality (Caverzasio *et al.,* 1998). On the other hand, fluorides anabolic activity induces uncoupling between bone resorption and formation which increases bone volume (Caverzasio *et al.,* 1998).

Farley *et al.* (1990) showed that clinically effective osteogenic fluoride concentrations of 5–30 µM were able to increase bone formation in vitro resulting from the enhanced activity of bone cell mitogens which led to an increase in osteoprogenitor cell proliferation. On the other hand, fluoride at a concentration of $10^{-5}$ M and $10^{-7}$ M was found to influence matrix metalloproteinases (MMPs) expression, thus affecting the remodeling matrix composition and bone mineralization (Waddington and Langley, 2003).

However, the longitudinal Iowa Fluoride study (Levy *et al.*, 2014) which measured the effects of life-long fluoride intake on bone measures of adolescents, concluded that fluoride exposures from fluoridated water (Fluoride concentration of water $\geq$ 0.7 ppm) in the US do not have any significant effect on bone mineral measures for the majority of adolescents.

Exposure to very high doses of fluoride over a long period of time could result in the development of skeletal fluorosis. Skeletal fluorosis is characterized by varying degrees of osteosclerosis, osteomalacia, osteoporosis and exostosis, results from the increased metabolic bone turnover, impaired bone collagen synthesis and increased avidity for calcium induced by fluoride toxicity (Lang, 2009; Krishnamachari, 1986).

The condition can affect both adults and children but an increased susceptibility is often associated with age, affecting the young and old. Younger children have an increased risk of fluoride bone accumulation due to their growing skeleton. On the other hand, the elderly often have a decreased rate of fluoride clearance from the kidneys (Bini, 2014). Dunipace *et al.* (1995) questioned the safety threshold of fluoride exposure with respect to age whilst considering the physiological changes in gastric secretion resulting from aging that may alter fluoride absorption. However, they found that the safety threshold of fluoride exposure did not change as populations' age.

Renal insufficiencies in general, increase the risk of skeletal fluorosis. However, those that are associated with other conditions such as diabetes mellitus also pose a risk and the severity of such susceptibility may be underestimated (Clement International Corporation, 2001).

The effects of skeletal fluorosis are observed after accumulation over a period of time i.e. dose and time dependant. Research claims that at least 10 mgF/day for ten years would be required to produce even the mildest form of skeletal fluorosis (Bernstein and Luggen, 2011; Gibney *et al.,* 2013). A study by Jolly *et al.* (1968) associated fluoride concentrations of 0.9 – 2.5 ppm to induce an incidence of only 2.4% skeletal fluorosis, whereas, they found crippling fluorosis to be associated with concentrations ranging from 1.3 to 5.2 ppm.

### 1.1.2. Teeth

1. **Cariostatic effect**

Dental caries is one of the most prevalent chronic diseases worldwide and a significant public health problem. In England, on average 12% of three-year olds (Public Health England[A], 2014) and 24.7% of five years olds (Public Health England[A], 2016) have experienced dental decay with 3.07 and 3.4 $d_3$mft (out of 20 teeth), respectively. Almost all or majority of adults worldwide will have experienced dental caries at some point in their life (Dye *et al.,* 2015; WHO, 2012; Steel and O'Sullivan, 2009).

Dental caries is characterised by demineralisation of the tooth enamel. It occurs as a process over time following the development of plaque which reduces the pH of the tooth environment. The presence of sufficient fluoride in saliva decreases the demineralisation of enamel when oral bacteria lower the pH of dental plaque. As well as prevention, fluoridation can also reverse tooth decay by enhancing remineralisation of tooth enamel when the pH rises (Duggan *et al*., 2008). All in all, the dynamic balance between pathological factors leading to demineralisation and protective factors that lead to remineralisation can often determine the outcome of dental caries (Featherstone, 2004).

The benefits of fluoride are achieved via two different mechanisms: systemic fluoride and topical fluoride. Systemic fluoride provides benefit before and after tooth eruption by binding into the enamel minerals (Rosin-Grget and Lincir, 2001) as well as ingested fluoride returning to the oral cavity via saliva (Buzalaf *et al*., 2011). Systemic fluoride is primarily received through water fluoridation as well as many other sources including fluoridated milk, salts and supplements. However, it is recommended that children under the age of 6 months should not receive fluoride supplements to avoid the excessive fluoride intake. Systemic fluoride exerts anti-caries effects during tooth formation and therefore children under the

age of 6 are at greater risk of fluorosis from excessive fluoride ingestion (Sampaio and Levy, 2011).

Topical fluoride, on the other hand, is applied directly to teeth and benefits erupted teeth. It comes from many sources including toothpaste, gel, mouthwash, varnishes, etc. Topical application of fluoride i.e. post-eruptive plays a dominant role in caries prevention in comparison to systemic fluoride. It lasts as long as fluoride exposure continues and is gradually lost after fluoride exposure is discontinued (Hellwig and Lennon, 2004).

The recommended dose of dentifrices for the anti-caries effect of topical fluoride is 1000 ppm and above (Walsh *et al.,* 2010). However, if the risk of fluorosis is of concern, then the fluoride concentration in the dentifrice can be lower than 1000 ppm (Wong *et al.,* 2010).

Post-eruptively topical fluoride utilizes a combination of mechanisms. It exerts anti-microbial activity by inhibiting bacterial virulence factors such as acid production and glucan synthesis of *S. mutans* (in vitro) and in addition to this, it plays a role in the de-mineralisation and re-mineralisation of dental hard tissue (Buzalaf, 2011). Aoba and Fejerskov (2002) stated that because the predominant cariostatic effect of fluoride is not due to its uptake by the enamel during tooth development but during cyclic de- and re-mineralization which takes place at the tooth, it is possible to achieve caries reduction without concomitant risk of dental fluorosis.

Considering that oral hygiene is one of the most protective factors as well as having to combat issues relating life-style and behaviour on a local scale, many public health interventions are constantly reviewed and put into place for prevention. This helps increase oral health awareness, educate families and most importantly administer oral hygiene practices, especially in schools (Watt, 2005; Weintraub, 2011).

The current treatment for dental caries varies with the severity and degree of damage. Progression of early stage dental caries can be prevented by the application of a fluoride varnish; however, this requires early diagnosis which does not always happen. In cases where most of the enamel has worn away leading to the formation of a cavity, the soft decay is removed and a filling is required. If left undiagnosed, this can lead to nerve damage which requires root canal treatment followed by either a filling or a crown. In the worst of cases, if the tooth cannot be restored, it is extracted and this sometimes requires hospitalisation. Almost 60,000 children and adolescents (aged 0 to 19 years) were admitted into hospital for extraction of one or more decayed teeth in 2015/16 (Public Health England, 2016[B]). This

initiates a major cost burden on the health care services, costing up to a maximum of £2430 per child (Pukallus *et al.*, 2013), making dental caries one of the costliest human diseases even more than cardiovascular disease and diabetes (Murray *et al.*, 2003).

According to Welbury *et al.* (2012) preventative measures indicative of good oral health and reduction of caries include: 1) Plaque control and regular tooth brushing with fluoride toothpaste; 2) Sensible dietary advice; 3) Use of fluorides; 4) Fissure sealants; 5) Regular dental checks with appropriate radiographs. Of these, use of fluoride is said to be the most effective treatment and prevention playing a significant role in dental caries (Carey, 2014). The prevalence of caries has significantly decreased over the years since the introduction of fluoridated interventions (American Dental Associations, 2005).

## 2. Dental Fluorosis

Although, fluoride at low levels prevents dental caries, excessive exposure to fluoride can lead to the development of dental fluorosis. Dental fluorosis is a chronic fluoride induced condition characterised by abnormal enamel development and hypo-mineralisation. The severity can range from mild to severe, with severe fluorosis leading to weak and rough teeth with yellow or brown patches on the surface (Sharma and Sharma, 2006).

The developing dentition is at most risk with the most prone being those at the post-secretary and early maturation phase of tooth development (Mascarenhas, 2000). Fluoride interacts with the mineralizing tissue causing porosity which is most likely initiated by a delay in enamel protein hydrolysis and removal (DenBesten, 1999). Different magnitudes of porosity at tooth eruption can be caused by interference with the enamel matrix removal which may produce impeding effects on the associated crystal growth during maturation (Aoba and Fejerskov, 2002).

A review conducted by Mascarenhas (2000) which quantified the risk factors associated with dental fluorosis from the 1980s to the 1990s, identified four major risk factors: fluoridated drinking water, fluoride supplements, fluoride tooth paste and infant formulas before the age of six. These risk factors are still the most important associated with dental fluorosis. Even though an increased exposure of fluoride is the highest risk factor, an increased susceptibility is posed with age. Children up to the age of 8 are prone to dental fluorosis with ages 3 - 6 being the most critical (Chaturvedi *et al.*, 2011). In contrast, it is also this age group which can be highly prone to dental caries if adequate measures are

not taken. Therefore, it is essential to ensure the balance between fluoride consumption for the prevention of caries and excessive fluoride exposure inducing fluorosis.

Many cases of fluorosis are so mild and difficult to detect, they are often undetected or only diagnosed upon dental check-up. The majority of treatments for more severe cases work mainly by bleaching the discolouration, abrasion, restoring the tooth, if possible; otherwise the use of veneers and crowns are employed (Sherwood, 2010). This also contributes to the cost burden associated with oral health and because the severity of dental fluorosis can be prevented, it is important to have the precautionary measures clearly defined. The prevention of dental fluorosis is largely dependent on parental vigilance. Awareness and knowledge of the various sources of fluoride intake along with the initiative of controlling intake e.g. from avoiding fluoride supplements if living in naturally high fluoridated area or then supervising the use of dentifrice, can all significantly reduce the risks (Alvarez *et al.*, 2009). Therefore, it is highly important that public health bodies, local authorities and dental practices work closely with families to ensure appropriate oral health education.

Measures can be put into place to minimise the over exposure to fluoride at both a public level and individual level. Browne *et al.* (2005) highlighted the age of fluoridated tooth brushing and amount of toothpaste as an important risk factor. They recommended that tooth brushing should not start before the age of 2 and a pea-sized amount (0.25 grams) of fluoridated toothpaste should be used. Parents play a major role in minimising the risks involved and it is recommended that tooth brushing should be supervised at all times. Dental practitioners should consider the fluoride content of the water supply for each individual before prescribing fluoride supplements (Mascarenhas, 2000).

## 1.2. Sources of Fluoride

Sources of fluoride intake vary, resulting from both natural and artificial forms that are readily available. They are mostly obtained from dietary sources as well as dentifrices and the exposure of such sources is often dependent on many factors such a geographical location, social class, lifestyle, dietary habits as well as age. This often makes it difficult to determine the total daily fluoride intake of a population and on an individual level, especially amongst younger children (Levy *et al.,* 1995). The average daily dietary fluoride intake of young children is estimated to be around 0.05 mg/kg body weight (Levy, 1994).

### 1.2.1. *Natural sources:*

The levels of fluoride detected in natural water can range from trace quantities to over 25 mg/l (Medical Research Council (MRC), 2002), however, these levels are influenced by the weathering of rocks and vary in geographical areas at the foot of high mountains and with deposits of marine origin (Tressaud and Haufe, 2008). Sometimes the levels of fluoride leaching into drinking water exceed optimal levels, ultimately posing a serious risk to human health. A study by Dissanayake (1991) found the natural fluoride content of ground water to exceed 5 mgF/l in parts of Sri Lanka which correlated with a high prevalence of dental fluorosis in those areas. Twenty-eight countries worldwide (around 280 million people) consume naturally fluoridated water with around 57.4 million people who receive it at the optimum level of 1 ppm. In the UK, areas of Hartlepool, Easington, Uttoxeter and Redbridge consisting of 330,000 people consume naturally fluoridated water at or around optimum levels (British Fluoridation Society, 2012).

As well as water, many foods naturally contain fluoride which contributes to fluoride intake from diet. In general, the fluoride content of such food groups is low, especially fruits and vegetables (<0.05 mg/100 g) (New Zealand Food Safety Authority (NZFSA), 2008). Some foods, however, can contain higher concentrations of fluoride and examples of such food include tea which contains 3.2 mgF/kg - 260 mgF/kg on average but can exceed up to 400 mgF/kg (Gupta & Sandesh, 2013). Marine fish like tuna, sardines and salmon which is sold canned and / or eaten with bones can end up with fish concentrates with up to 370 $mgF/kg^{-1}$ (Fawell *et al.*, 2006; Chichester and Stewart, 1982).

Although the fluoride content of most foods is low, preparation of food or beverages / infant formulas using fluoridated water can significantly increase the fluoride content. This was illustrated by Marier and Rose (2006) who found an average fluoride increase of 0.50 ppm when foods / beverages were prepared using fluoridated water at 1.0 ppm. According to this, foods prepared with fluoridated water concentrations > 1.0 ppm may have even greater increases in their fluoride content.

### 1.2.2. Artificial sources:

**1. Water**

The preventative role of fluoride in dental caries has enforced the implementation of water fluoridation as a public health measure for over 40 years (Public Health England, 2014). It is recognised as the most cost-effective method of reaching the whole population via piped water supplies, especially deprived communities and those at higher risk e.g. children and lower social class (MRC, 2002; Peterson and Lennon, 2004). The most common fluoride compounds used in water fluoridation are hydro fluorosilicate (FSA), sodium fluorosilicate and NaF (National Research Council (NRC), 2007; Buzalaf, 2011). In the UK, around 10-12% of the population receives fluoridated water, naturally or artificially adjusted to 1 ppm (Public Health England, 2014; British Fluoridation Society, 2010). According to a survey conducted by Public Health England in 2009 and 2012, children were less likely to have tooth decay by the age of 5 or 12 with fewer missing and filled teeth if living in fluoridated areas of England in comparison to those living in non-fluoridated areas (Public Health England[B], 2014).

Optimum levels of fluoride in fluoridated water under fluoridation schemes range between 0.7 – 1.2 ppm. Fluoridated water is one of the significant contributors of total daily fluoride intake in young children. This results from the consumption of water directly, as well as, fluoridated water used to prepare food and drink, thus, increasing the risk of fluorosis. It is therefore recommended that water with fluoride concentrations of less than 0.5 ppm should be used to prepare infant formulas for young children and infants (Buzalaf and Levy, 2011).

**2. Milk**

NaF and disodium monofluorophosphate are often used to supplement milk with the rationale that their ingestion increases the salivary fluoride concentration levels comparable to those detected for optimally fluoridated water (Buzalaf, 2011). Fluoridated milk schemes have also been incorporated as public health interventions, with over 40,000 school children now receiving fluoridated milk in the UK (Rajpura and Donkin, 2014). The costs associated with milk fluoridation in schools are estimated to be around £1.25 per child per year (Woodward *et al.*, 2008) and this is deemed to be a cost-effective measure when considering the risk benefit ratio. It is a cost-effective alternative to water fluoridation in areas where other preventative measures are not yet feasible (Meyer-Lueckel and Paris, 2013).

Milk fluoridation along with reconstituted milk formulas prepared using fluoridated water also contribute to the total daily fluoride intake of young children. They serve both topical benefits as well as systemically returning to the mouth via saliva. Therefore, the levels of fluoride in plasma, saliva and fluoridated milk are all linked. Several studies have challenged the significance of milk fluoridation in reducing dental caries and low evidence has been generated on the effectiveness of milk fluoridation (Cagetti *et al.*, 2012).

3. **Salt**

Another widely used alternative vehicle for fluoride that largely contributes to our diet is salt (potassium fluoride and NaF) (Buzalaf, 2011) which, like milk, protects the right of consumer choice. It has been recommended that a salt fluoride concentration of 250 µg/g does not pose any risk of dental fluorosis (Bergmann & Bergmann, 1995).

The cariostatic potential of salt fluoridation was comparable to that of water fluoridation in a review by Marthaler (2013), finding up to 50% in caries reduction. The comparability of fluoridated salt with water fluoridation had been supported by many researchers (Wesp and Burgi, 1971; Marthalet *et al.*, 1978).

The use of salt as a vehicle for fluoridation has a few limitations. The increased use of salt is associated with hypertension and people at high risk of hypertension may be at risk along with not being able to benefit from the anti-caries effect of fluoridated salt if alternative diets are employed. Therefore, alternative means of fluoride supplementation should be provided to such individuals (Bergmann and Bergmann, 1995).

4. **Toothpaste and Dental products:**

The other major source of systemic fluoride is dental products such as fluoridated toothpaste, mouth wash, dental floss, etc. This results from the unintentional ingestion of toothpaste and other dental products due to the development of the spitting reflex, especially in younger children. The ideal concentration of fluoride in toothpaste suitable for family use is commonly around 1000 ppm in compliance with the current international standard level recommendation (Walsh *et al.,* 2010). The benefit of having one concentration to target caries prevention for all ages arises from the elimination of costs associated with the purchase of several different concentrations for one household, which highly benefits populations with lower education or poverty.

Although a fluoride toothpaste concentration of 1000 ppm is sufficient for caries prevention, higher concentrations ranging 4000 – 5000 ppm are available through prescription for those at higher risk of tooth decay e.g. based on clinical assessment of tooth health, etc. (Clark and Slayton, 2014). Dental practitioners also employ the use of fluoride varnishes for high risk children and Carey (2014) stated the recommendation of fluoride varnish application twice a year to be sufficient for caries prevention in permanent teeth. As well as professionally applied fluoride, dental practitioners can also prescribe fluoride supplements in the form of tablets, lozenges, etc. however, a major disadvantage arises from individual compliance which is a significant problem in underprivileged populations (Espelid, 2009).

## 1.3. Fluoride Metabolism

Fluoride in its many sources, is ingested through the oral cavity and absorbed mainly through the stomach. Following absorption, it is distributed throughout the body via plasma and bodily fluids upon which some is retained, and the rest is excreted.

### 1.3.1. Absorption

Fluoride ingestion starts in the oral cavity where absorption levels are low. Fluoride absorption mainly occurs via the gastrointestinal tract. Hydrogen fluoride (HF) occurs in the acidic environment of the stomach due to the increased coefficient permeability of lipid bilayer membranes to HF, in comparison to $F^-$. This allows HF to readily cross the cell membranes in response to the pH gradient between adjacent bodily fluid compartments (Buzalaf, 2011; SCHER, 2011). Up to 40% of HF is absorbed in the stomach and up to 45% from the small intestine (Barbier *et al.,* 2010).

Absorption from the stomach is pH dependent with a lower pH favouring the rate of absorption via passive diffusion. Absorption via the small intestine, on the other hand, is not pH dependant and occurs as the ionic form rather than HF, as described in the stomach (Tressaud and Haufe, 2008; Buzalaf, 2011).

The degree of fluoride absorption can vary and depends on the form of fluoride being consumed. Highly soluble fluoride compounds such as sodium fluorosilicate (found in fluoridated water) and NaF or monofluorophosphate (found in toothpastes) are almost absorbed 100% (Gropper *et al.,* 2008). Compounds with lower solubility are less well

absorbed, such as calcium fluoride, magnesium fluoride and aluminium fluoride. The absorption of fluoride can also be affected by the intake of certain foods, especially solid foods and those with high calcium content e.g. dairy products or milk. Shulman and Vallejo (1990) found that consuming fluoride with solid foods and milk reduced fluoride absorption by 47% and 13%, respectively.

### 1.3.2. Distribution

Once absorbed, fluoride is rapidly distributed by circulation to the intracellular and extracellular fluids. Such rapid uptake allows peak plasma fluoride levels to be reached within 20 – 60 minutes post ingestion (Whitford, 1994) before levels start to decline. Fluoride concentrations of several bodily fluids are related to plasma levels in a steady state manner, such as urine, bile, ductal saliva and gingival crevicular fluid (NRC, 1993). Plasma fluoride concentrations are not homeostatically controlled but largely influenced by fluoride intake. The rate of bone accretion and dissolution also plays a role in the level of plasma fluoride along with the renal clearance rate (SCHER, 2011).

### 1.3.3. Excretion

The absorbed fluoride is mainly excreted via urine with little also being excreted by faeces. Fluoride is filtered from the plasma via the glomerulus. Some of the fluoride is reabsorbed, whilst there is no tubular secretion of fluoride. The rate of urinary fluoride excretion is influenced by several factors including glomerular filtration rate, urinary pH and urinary flow (SCHER, 2011).

Urinary fluoride clearance increases with urinary pH due to a decrease in the concentration of HF. Various factors can affect urinary pH and thus affect fluoride clearance and retention (Agency for toxic substances and disease registry (ATSDR), 2003). Dietary factors play a major role in determining urinary pH. Consuming large quantities of foods that lead to low urinary pH increase the rate of urinary fluoride excretion. An example of such foods includes citrus fruits, vegetables and dairy products.

The renal clearance rate of children is around 45 ml/min (Spak *et al.,* 1985) and of adults is around 30 to 50 ml/min (Marya, 2011). Renal insufficiency could decrease the rate of fluoride clearance, thus, enhancing chances of skeletal fluorosis. Even the slightest renal

impairment could lead to excessive fluoride retention and therefore increase the susceptibility to skeletal fluorosis due to less fluoride being excreted via urine and thus increasing fluoride retention.

### 1.3.4. Retention

Once distributed, approximately 99% of absorbed fluoride is retained in calcified tissue i.e. mainly bone but also dentine and enamel. The amount of fluoride retained in infants (80-90%) is higher than that in adults (60%) (Barbier *et al.,* 2010) resulting from a growing skeleton. When being filtered in the kidneys, some fluoride is partially reabsorbed and retained; therefore, a higher concentration of fluoride is often reported in the kidneys than other soft tissue (Whitford, 1996).

The amount of fluoride retained is dependent on fluoride intake and excretion. There is an increase in retention when fluoride intake is greater than excretion (positive balance). Likewise, the rate of retention is reduced when fluoride intake is less than fluoride excretion (negative balance).

## 1.4. Study Rationale and Gaps in literature

Dental caries remains a substantial global public health problem affecting on average 12% of three-year olds and 30% of five-year olds (Public Health England (PHE)[A], 2014; PHE, 2016). Considering the preventative role of fluoride in dental caries, public health initiatives such as fluoridated milk schemes have been rolled out across schools. Around 40,000 school children now receive fluoridated milk (Rajpura and Donkin, 2014). However, previous research has indicated that the current UK milk fluoridation scheme does not provide adequate protection for prevention of dental caries (Ketley and Lennon, 2000).

Knowledge around fluoride metabolism is limited and there is a lack of understanding relating the effects of physical activity on fluoride metabolism within the human body. However, the collection of fluoride biomarkers used to measure fluoride metabolism, especially blood, can be invasive, unethical and pose the risk of harm. This can be a major drawback, especially when involving young children.

There is a direct relation between bone and plasma fluoride (Carvalho *et al.,* 2006) and subsequently a proportional relation between plasma and parotid / submandibular ductal salivary fluoride concentrations (Oliveby e*t al*., 1989). Therefore, there is need for the search of other bodily fluids which can be collected in a novel, simple, practical and less invasive alternative to plasma for fluoride studies.

With respect to the effects of exercise on fluoride metabolism, a study in rats (Whitford, 1989) found a significant low and delayed peak plasma fluoride concentrations during lightly exercised compared with non-exercised rats. The authors also reported significantly higher plasma fluoride in non-exercised rats (58% to 76%) at different time points, up to 3 hours after exercise.

Reduced fluoride plasma levels have also been associated with exercise in rats in other studies (further discussed in section 2.6), (Basha and Sujitha, 2012; Lombarte *et al.,* 2013) however, it is known that rats metabolise fluoride around ten times faster than humans and for comparison 15 mg/l NaF is equivalent to 3 mg/l in humans (Dunipace *et al*., 1995; Lombarte *et al*., 2013; Lima *et al.,* 2014). Therefore, although, there is lack of evidence of any such effects in humans, findings from rats can be used to provide the basis of further investigation in human subjects.

The main hypothesis of this project is that exercise may affect the metabolism of fluoride in humans. The main novelties of the project will also arise from the findings of the effects of exercise on fluoride metabolism. It is hypothesised that the timing of physical exercise following the distribution of school milk could have an impact on the effectiveness of the programme in the control of dental caries. The anti-caries effect of fluoridated milk is mainly topical and instantaneous, as mentioned previously (section 1.2.2) with rotating benefits and exercise in anticipated to disrupt the impact and therefore, potentially leading to greater retention in the body with less distributed in the mouth via saliva.

Further understanding the mechanisms of fluoride metabolism may help to maximise the beneficial effects of fluoride for caries prevention, while minimising the risk of dental fluorosis.

## 1.5. Outline of Thesis

This thesis is divided into 10 chapters. Chapter 1 provides an introduction and study background followed by Chapter 2 which presents a review of literature on the current knowledge around fluoride metabolism and exercise relevant to this study. Chapter 3 lays out the aims and objectives of the four studies involved. The first study i.e. Practicality and suitability of Dry Tips and Lashley Cups for the collection of saliva: A Preliminary Investigation, is presented in Chapter 4. Findings from Study 1 initiated the development of the methods used in Study 2, which is presented in Chapter 5: Determination of fluoride in saliva and plasma: sample collection and analysis.

Overall findings from Study 1 and 2 helped to finalise the method of fluoride sample collection to be used in the main study of this thesis (Study 3): The effects of different intensities of continuous exercise on fluoride metabolism in adult humans, in Chapter 6. Study 3 then provided the basis for a similar study aiming to develop a practical and ethically approved protocol to investigate the effects of exercise on fluoride excretion in children which is presented in Chapter 7 (Study 4). Chapter 8 provides an overall discussion of the four studies involved along with drawing conclusions and recommendations for future work. The overall link between studies in this project is illustrated by Figure 1.1. Finally, references and appendices can be found in Chapters 9 and 10 (part 2), respectively.

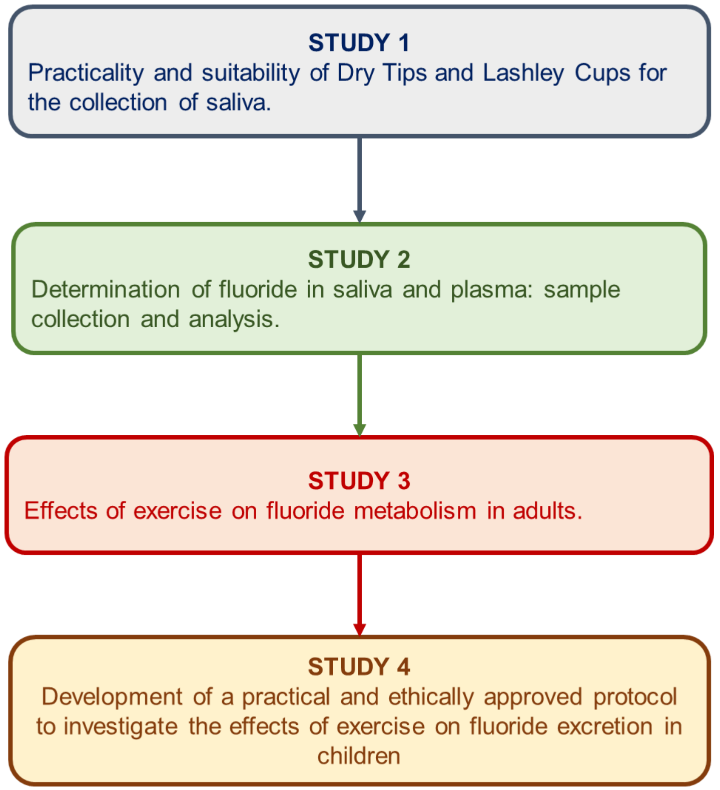


**Figure 1.1. Overall link between the studies in this project.**

# Chapter 2. Literature Review

## 2.1. Introduction

This chapter presents a critical literature review covering areas of fluoride metabolism and factors affecting fluoride metabolism with a particular focus on physical activity. It also reviews topics of methods for collection of biological samples such as urine, blood and saliva; as well as relevant analytical methods for determining fluoride in different biological samples.

## 2.2. Metabolism of fluoride

### 2.2.1. Fluoride Ingestion

Main sources of systemic fluoride ingestion include diet (including food, water and beverages), dental products and supplements. Generally, the greatest contributor of total daily fluoride intake could be water, either directly as a drink or then used to prepare/cook food and drink. Fluoride delivery methods can be divided into three categories: 1) Community based i.e. fluoridated water, salt and milk; 2) Professionally administered i.e. fluoride gels and varnishes; and 3) Self-administered i.e. toothpaste and mouth-rinses (O'Mullane *et al.,* 2016).

Around 369,226,000 people across 25 countries were estimated to receive artificially fluoridated water in the year 2011. Of them 25 countries, 18,061,000 million people were drinking naturally fluoridated water at or around the optimal level and a further 239,903,000 million people from 27 other countries were also receiving naturally fluoridated water (O'Mullane *et al.,* 2016).

Fluoride levels in drinking water in the range of 1 – 1.5 mg/l can strengthen enamel. Levels of 1.5 – 4 mg/l can induce dental fluorosis and higher levels of 4 – 10 mg/l can progress dental fluorosis to skeletal fluorosis (Mohapatra *et al*., 2009). In order to minimise the risk of high fluoride exposure, it is important to ensure safe levels of fluoride in drinking water. Many health authorities have recommended optimum levels and safety thresholds of water fluoridation to minimise these risks as well as allowing the beneficial effects of fluoride at the same time. In non-fluoridated areas, where the prevalence of dental caries is high or increasing, the local water supplies can be artificially fluoridated. Table 2.1 presents an example of the optimum fluoride levels in drinking water recommended by health authorities

in different countries. The optimal levels vary for each country, depending on the temperature of the climate and subsequently water intake, for example, an increased water intake is witnessed in countries with a warmer climate (U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation, 2015). However, a study by Beltrán-Aguilar *et al.* (2015) found that water intake was not associated with increased temperature. They recommended that the optimal fluoride concentrations in drinking water to prevent dental caries does not need to be based on outdoor temperature. Khan *et al.* (2004) recommended that it should be based on the dose-response relationship of fluoride in drinking water with the levels of caries and fluorosis, whilst considering climatic conditions, dietary habits and other sources of fluoride exposure, for any given population. These findings will impact future fluoridation schemes and policies for many countries worldwide.

**Table 2.1. The optimum fluoride levels of fluoride in drinking water in different countries. (adapted from Peckham, 2012).**

| Country | Recommended fluoride levels (ppm) |
|---|---|
| UK | 1.0 |
| Canada | 0.7 |
| Eire | 0.7 |
| Hong Kong | 0.5 |
| US | 0.7 |

The use of fluoridated water to prepare meals, beverages and infant formulas can greatly increase the level of fluoride ingested. The levels of fluoride in food and beverages vary widely, ranging from as low as <0.01 µg F/100 g for butter/margarine to as high as 1,054.20 µg F/100 g for canned sardines (Zohoori and Maguire, 2016). The fluoride levels in some food and beverages are presented in Table 2.2. The variation in fluoride concentrations for the same item can result from fluoride pollution of the air by industries; the humidity of the

climate; fluoride-containing fertilizers and pesticide residues; the type of soil for plant growth; and the methods of industrial processing and home preparation (Kumpulainen and Koivistoinen, 1977). This can ultimately affect the amount of fluoride which is ingested by each individual living in the same community, regardless of the water fluoride levels of that area.

**Table 2.2. Fluoride levels in food items and beverages. (adapted from Miziara *et al.* (2009) and Zohoori and Maguire (2016)).**

| **Food Group** | **Food / Beverage** | **Level of fluoride (µg/100g)** | |
|---|---|---|---|
| | | **Miziara *et al.* (2009)** | **Zohoori and Maguire (2016)** |
| **Fruit** | Banana | 1.00 | 0.77 |
| | Apple | 1.80 | 1.90 |
| | Pear | 1.80 | 1.80 |
| | Orange | 0.40 | 2.30 |
| | Papaya | 1.10 | * |
| **Vegetable** | Carrot | 0.70 | 0.95 |
| | Pumpkin | 3.50 | * |
| | Spinach, Cabbage & Kale | *5.00* | * |
| | Lettuce | 2.50 | 5.45 |
| | Tomato | 2.00 | 1.05 |
| **Meat** | Beef | 3.00 | 5.60-5.80 |
| | Chicken | 9.60 | 2.50-3.20 |
| | Ham / salami | 9.50 | 6.93 |
| | Sausage | 65.80 | 1.60 |
| **Dairy** | Whole milk | 1.50 | 0.80 |

| | | | |
|---|---|---|---|
| | Powdered milk (prepared using 0.6-0.8 ppm F water) | 141.49 | * |
| | Fermented milk | 13.20 | * |
| | Infant milk formula | * | 0.90-25.20 |
| | Fruit yogurt | 86.0 | 3.60 |
| | Butter<br>Margarine | 68.2<br>68.2 | 0.05<br>0.00 |
| | Cheese | 6.80 | 8.25 |

**Table 2.2. continued**

| Food Group | Food / Beverage | Level of fluoride (µg/100g) | |
|---|---|---|---|
| | | Miziara *et al.* (2009) | Zohoori and Maguire (2016) |
| **Other food** | Rice<br>(boiled using 0.79-0.99 ppm F water) | 11.20<br>* | *<br>175.20 |
| | Baked potato | 14.20 | 14.00 |
| | Biscuits (non-filled)<br>(filled) | 91.40<br>3.30 | 4.20-21.00<br>9.80 |
| | Cereal | 164.00 | 3.90-75.30 |
| | Pasta | 15.54 | * |
| | Noodles<br>(boiled using<br>(boiled using 0.05-0.99 ppm F water) | 51.10<br>* | *<br>10.75-41.30 |

| | | | |
|---|---|---|---|
| | Bread (White)<br>(Brown)<br>(wholemeal) | *<br>*<br>* | 56.10<br>22.20<br>22.20 |
| **Sweets / Snacks** | Cream cheese | 12.90 | * |
| | Sugar<br>(unspecified)<br>(White)<br>(brown) | <br>20.88<br>*<br>* | <br>*<br>1.20<br>23.95 |
| | Cake | 25.30 | 5.40-26.0 |
| | Chocolate bar | 11.00 | 6.40-8.20 |
| | Powdered chocolate | 21.70 | 23.0 |
| | Potato crisps | 31.80 | 23.80 |
| **Beverages** | Tea (unspecified)<br>Black tea, infused using 0.05-0.99 ppm F water) | 33.50<br>302.10 | *<br>60.70-193.30 |
| | Coffee<br>(sugared)<br>(prepared using 0.05-0.99 ppm F water) | <br>18.49<br>* | <br>*<br>7.00-83.00 |
| | Powdered juice | 25.80 | * |
| | Orange juice | 0.40 | 2.30 |
| | Other fruit juices | 0.60 | * |
| | Fruit juice drink | * | 4.80-10.20 |
| | Soft drink | 24.00 | 10.50 |

Note: * Not Available

#### 2.2.1.1. Fluoride ingestion in children

Zohouri *et al.* (2006) investigated the relative contributions of different dietary sources to dietary fluoride intake and compared this in children aged 6 years (n= 33) from optimally artificially fluoridated (0.7-1.0 mgF/L), sub-optimally artificially fluoridated (>0.3 to <0.7 mgF/L), and non-fluoridated areas (<0.3 mgF/L) in the UK. They collected dietary data using a 3-day dietary diary along with analysing the fluoride content of food and drink samples that were consumed by participants. They found the constituents of diet that contributed to dietary fluoride intake to vary between fluoridated and non-fluoridated areas. In fluoridated areas, they found tap water, fruit squashes and cordials prepared with tap water, as well as cooked rice, pasta and vegetables to be the most important contributors of fluoride intake. Whereas in non-fluoridated areas, carbonated soft drinks and bread were found to be the most important contributors to dietary fluoride intake. Overall, drinks were responsible for 59%, 55% and 32% of dietary fluoride intake in optimally, sub-optimally and non-fluoridated areas respectively.

A similar study was conducted in Iran, in which sources of dietary fluoride intake were estimated in children aged 4 years (n= 103) in three areas with mean fluoridated water levels of 0.3, 0.6 and 4.0 mgF/l (Zohouri and Rugg-Gunn (2000)[A]). They found mean dietary fluoride intakes of 413, 698 and 3472 ug/day, respectively. Drinks were responsible for 72 - 87% of dietary fluoride and this range increased with an increase in water fluoride concentration and climate temperature. Although water contributed more than soft drinks, of the drinks in general, they found tea (infused) to be the greatest contributor, responsible for 31 - 38 % of total dietary fluoride intake. Like Zohouri *et al.* (2006), they also found cooked rice and bread to be the greatest food sources of fluoride, again increasing as the water fluoride concentration increased.

In population with tooth brushing habits, diet and dentifrice combined are both significant contributors to total daily fluoride ingestion. Many food and beverages naturally contain fluoride which contributes to the total daily fluoride intake. In children the majority of ingested fluoride may arise from dentifrice and dental products which are ingested unintentionally. Zohoori *et al.* (2014) measured the total daily fluoride intake (TDFI) of infants aged 1 - 12 months, living in non-fluoridated (0.19 mgF/l) (n= 19) and fluoridated (0.97 mgF/l) (n= 19) areas in north-east England. They used the 3-day dietary diary to assess dietary fluoride intake along with measuring the fluoride content of food and drink samples consumed by participants. Data on tooth brushing habits was gained by a questionnaire

with an interview. They estimated TDFI from diet and dentifrice ingestion. Mean (SD) TDFI for the infants was found to be 0.024 (0.015) mg/kg body weight per day in non-fluoridated areas and 0.107 (0.054) mg/kg body weight per day in fluoridated areas. Dentifrice contributed to 24 – 85% of TDFI for infants with tooth brushing habits but at a very young age of <1 year, tooth brushing habits were only reported for 5 infants. Therefore, diet was reported as the main contributor of TDFI in infants in this study with it being the only source of fluoride for 87% of infants.

Almeida *et al.* (2007) investigated fluoride ingestion from diet and toothpaste in 1- to 3-year-old children (n= 33) and found dentifrice to contribute to an average 81.5% of the total daily fluoride intake which is within the range reported by Zohoori *et al.* (2014). The amount of toothpaste retained in the mouth of young children aged 30 months (n= 50) was found to be around 72% with a mean fluoride ingestion of 0.06 mgF/kg/day for those brushing with a family toothpaste (1450 ppm) twice a day (Bentley *et al.*, 1999).

The use of dental products leading to the unintentional ingestion of fluoride arises due to the spitting reflex, especially in younger children. Dentifrice ingestion gradually starts to decrease to around 12% by the age of 8 - 10 years (Ellwood *et al.,* 2008), mostly due to the development of a mature spitting reflex. This puts younger children who are prone to dental fluorosis at greater risk of exceeding the optimal daily total fluoride intake.

Using fluoridated toothpaste before the age of 6 has been observed as a risk indicator for fluorosis and for children with fluorosis beginning brushing before the age of 2 years has been found to significantly increase the severity of fluorosis (Mascarenhas and Burt, 1998). Therefore, it is highly recommended that young children should be supervised whilst brushing twice a day with a pea-sized amount (0.25 grams (g)) of fluoride toothpaste whilst encouraging spitting and avoiding rinsing, in order to balance the benefits and risks associated (Davies *et al.,* 2003).

A study by Zohoori *et al.* (2012) measured the amount of fluoride that is ingested from toothpaste by 4-6-year-old children from low socio-economic areas (n= 38) and high socio-economic areas (n= 23), and the effects of age, gender and social class on the amount of fluoride ingested. They analysed all expectorated saliva samples, rinse water (if used) and residual toothpaste after brushing at home. Not all children brushed twice a day (in accordance to current guidelines), 69% of children from low socio-economic areas and 74% of children from high socio-economic areas reported brushing twice a day. Although, this

would minimise the risk of excessive fluoride ingestion from dentifrice for those not brushing twice a day, it would also affect the amount of caries protection that they could receive.

The mean (SD) amount of fluoride ingested was 17.0 (14.7) µg/kgbw/day and 29.3 (32.8) µg/kgbw/day, respectively, regardless of socio-economic areas. The mean (SD) weight of toothpaste dispensed was found to be 0.67 (0.36) g which is almost twice the recommended amount of a pea sized (0.25 g) amount of toothpaste.

Almeida *et al.* (2007) estimated the total daily fluoride intake of 1- to 3-year-old children (n= 33) from diet and dentifrice and found dentifrice to contribute to an average of 81.5% of the daily fluoride intake. Mean (±SD) fluoride intake from diet and dentifrice was 0.025 ± 0.013 and 0.106 ± 0.085 mg/kgbw/day, respectively. Of diet, water and milk were found to be the greatest contributors of fluoride. de Almeida *et al.* (2007) found the average amount of toothpaste dispensed to be 0.49 g which is again almost twice the amount of the recommended 0.25 g of pea sized amount.

Maguire *et al.* (2007) measured total fluoride intake, urinary fluoride excretion and estimated fluoride retention in children aged 6 - 7 years, living in either sub-optimally fluoridated (0.47 mgF/l) (n= 9), optimally fluoridated water (0.82 mgF/l) (n= 6) or non-fluoridated (0.08 mgF/l) (n= 18) areas. They collected diet and tooth brushing data using a 3-day dietary diary and analysed the fluoride content of all food and drink samples consumed by participants along with expectorated saliva during tooth brushing. They found mean fluoride intake from diet and toothpaste of 0.031 (±0.025) mg/kgbw/day for the low-fluoride area, 0.038 (±0.038) mg/kgbw/day for the sub-optimally fluoridated area and 0.047(±0.008) mg/kgbw/day for optimally fluoridated areas. Toothpaste was found to contribute to 57%, 35% and 47% of total fluoride intake for children receiving low, sub-optimally and optimally fluoridated water, respectively.

Such findings suggest that intake from all sources of fluoride exposure (predominantly toothpaste) have a major impact on fluoride retention in the body, particularly in low fluoridated areas and thus increase the risk of exceeding safe fluoride limits. Several studies which investigated the levels of fluoride ingestion from diet, dentifrice and water found ingestion levels to exceed safety thresholds of 0.05 - 0.07 mg F/kgbw/day (Paiva *et al.* (2003); Rodrigues *et al.* (2009); Lima and Cury (2001)) as summarised in Table 2.3.

A study by Martins *et al.* (2008) investigated whether there was a relationship between fluoride intake and dental fluorosis amongst young children aged 19 - 39 months living in

fluoridated areas (0.6 - 0.8 ppm). They found no association between dental fluorosis in permanent teeth and fluoride intake from diet, dentifrice or both combined. However, a major limitation of this study is that fluoride intake was only measured on one occasion and further work is required to support these findings.

Miziara *et al.* (2009) investigated the fluoride intake of 2–6-year-old Brazilian children (n= 379). Diet and dentifrice were found to contribute to 43.7% and 56.3%, of the total daily fluoride intake, respectively. However, 31.2% of the children evaluated were considered at risk to develop dental fluorosis as fluoride ingestion levels exceeded the optimum level of 0.07 mg⁄kgbw/day, thus increasing fluoride retention.

Although, young children are susceptible to ingesting excess fluoride from diet and dentifrice and consequently retaining a high proportion of ingested fluoride, previous findings have also reported low retention of fluoride in children. This was found in a study measuring total fluoride intake and urinary excretion in 4-year-old Iranian children (n= 78) residing in low-fluoride areas (0.30 - 0.39 mgF/l) (Zohouri and Rugg-Gunn, 2000)[B]. They found mean ingestion of fluoride from all sources to be 0.426 mg/day and mean fluoride urinary excretion to be 0.339 mg/day. The difference between ingestion and urinary excretion was +0.087 mg which is equivalent to 80% excretion. The greater excretion than ingestion suggested that fluoride retention was low. Zohouri and Rugg-Gunn (2000)[B] suggested this may have occurred due to the vegetarian diet of Iranian children which would lead to more alkaline urine and consequently increased fluoride excretion.

#### 2.2.1.2. Fluoride ingestion in adults

Even in adults, beverages and drinking water have found to be the greatest source of dietary fluoride intake. Singer *et al.* (1985) measured the daily dietary fluoride intake of 15 - 19-year-old males from non-fluoridated (<0.3 ppm) and fluoridated (0.7 - 1.0 ppm) areas in the US. They found an average daily dietary fluoride intake of 0.86 mg/day for non-fluoridated areas and 1.85 mg/day for fluoridated areas. Of the daily dietary fluoride intake, beverages and drinking water alone contributed of 75%. Levels of daily dietary fluoride intake in Canadian adults were found to be 0.56 mg/day and 2.80 mg/day living at areas with water fluoride of 0.0002 mg/l and 0.001 mg/l (Dabeka *et al.,* 1987).

During tooth brushing, adults only ingest less than 10% of toothpaste. Fluoride ingestion from toothpaste and dentifrice is only responsible for 5% and 8% of the total daily fluoride

intake, respectively (Villa *et al.,* 2004). In the majority of studies measuring fluoride intake from adults (Table 2.3 B), the main sources of ingestion are water, diet and supplements (Singer *et al.* (1985); Peckham (2012); Dabeka *et al.* (1987); Villa *et al.* (2004); Villa *et al.* (2008); Spencer *et al.* (1970); Spencer *et al.* (1975)[A]; Maheshwari *et al.* (1981)).

Levels of fluoride ingestion from various sources of fluoride have been summarised for children (Table 2.3 A) and adults (Table 2.3 B).

**Table 2.3. Fluoride ingestion levels.**

**A. Fluoride ingestion levels in children.**

| Age (years) (N) | Country | Fluoride concentration of water (mg/l) | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 6 (33) | UK | <0.3<br>>0.3 - <0.7<br><0.7 | 0.188 [b]<br>0.349 [b]<br>0.591 [b] | *<br>*<br>* | *<br>*<br>* | Zohouri *et al.* (2006) |
| 6 - 7 (33) | UK | 0.30<br>1.06 | * | * | 0.038[a]<br>0.076 [a] | Zohoori *et al.* (2013) |
| 0.1 - 1 (38) | UK | 0.19<br>0.97 | * | 0.20 – 0.50 [b]<br>0.10 – 0.50 [b] | 0.024 [a]<br>0.107 [b] | Zohouri *et al.* (2014) |
| 4 - 6 (61) | UK | 0.0009 | * | 0.029 [a] | * | Zohoori *et al.* (2012) |

**Table 2.3 A continued**

| Age (years) (N) | Country | Fluoride concentration of water (mg/l) | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 6 - 7 (33) | UK | 0.08<br>0.47<br>0.82 | 0.008 [a]<br>0.016 [a]<br>0.025 [a] | 0.023 [a]<br>0.022 [a]<br>* | 0.031 [a]<br>0.038 [a]<br>0.047 [a] | Maguire *et al.* (2007) |
| <4 (15)<br>(14) | Brazil | 0.04<br>0.64 | 0.006 - 0.011 [a]<br>0.011 - 0.015 [a] | 0.055 [a]<br>0.037 [a] | 0.011 - 0.061 [a]<br>0.015 - 0.048 [a] | Zohoori *et al.* (2013) |
| 1 - 3 (33) | Brazil | 0.6 - 0.8 | 0.31 [b]<br>0.025 [a] | 1.34 [b]<br>0.106 [a] | 0.130 [a] | Almeida *et al.* (2007) |
| 1.7 - 3.2 (71) | Brazil | 0.6 - 0.8 | 0.027 - 0.040 [a] | 0.052 - 0.061 [a] | 0.088 - 0.090 [a] | Paiva *et al.* (2003) |
| 1.7 (29) | Brazil | 0.6 - 0.8 | 0.031 [a] | 0.051 [a] | 0.083 [a] | Martins *et al.* (2008) |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3.3 (30) | | | 0.029 [a] | 0.049 [a] | 0.084 [a] | |
| 1.8 - 2.6 (39) | Brazil | 0.6 - 0.8 | * | * | 0.090 [a] | Lima and Cury (2001) |

**Table 2.3 A continued**

| Age (years) (N) | Country | Fluoride concentration of water (mg/l) | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 2 – 6 (398) | Brazil | 0.6 - 0.8 | 0.027 [a] | 0.036 [a] | 0.064 [a] | Miziara *et al.* (2009) |
| 4 - 6 | | | | | | |
| (24) | | Non-fluoridated | * | * | 0.01 [a] | |
| (25) | Brazil | 0.6 - 0.8 | * | * | 0.04 [a] | Rodrigues *et al.* (2009) |
| (21) | | 0.6 - 0.9 | * | * | 0.06 [a] | |
| (26) | | 180-200 mg/kg (salt) | * | * | 0.06 [a] | |
| 4 (103) | Iran | 0.3 | | * | * | Zohouri and Rugg-Gunn (2000)[A] |
| | | 0.6 | 0.413 [b] | * | * | |
| | | 4.0 | 0.698 [b] | * | * | |

| | | | 3.472 [b] | | | |
|---|---|---|---|---|---|---|

**Table 2.3 A continued**

| Age (years) (N) | Country | Fluoride concentration of water (mg/l) | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 4 | Iran | 0.30 - 0.39 | | | | Zohouri and Rugg-Gunn (2000)[B] |
| (32) | | | 0.023 [a] | 0.007 [a] | 0.030 [a] | |
| (28) | | | 0.026 [a] | 0.0008 [a] | 0.028 [a] | |
| (10) | | | 0.039 [a] | * | 0.039 [a] | |
| (8) | | | 0.029 [a] | * | 0.029 [a] | |
| * | US | 0.7 - 1.2 | * | * | 0.9-3.6 [b] | Peckham (2012) |

Note:

* - Data not available

[a] - mg/kg bw / day

[b] – mg/day

**B. Fluoride ingestion levels in adults.**

| Age (N) | Country | Water fluoride (mg/l) [fluoride source] | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 15 - 19 | US | <0.3<br>>0.7 | 0.86 [a]<br>1.85 [a] | *<br>* | *<br>* | Singer *et al.* (1985) |
| * | US | 0.7 - 1.2 | * | * | 1.58 - 6.6 [a] | Peckham (2012) |
| (24) | Canada | 0.0002<br>0.001 | 8.5 [b]<br>39.7 [b] | *<br>* | *<br>* | Dabeka *et al.* (1987) |
| 18 - 75 (115) | Chile | * | * | * | 0.53 - 2.97 [a] | Villa *et al.* 2010 |
| 19 - 73 (73) | Chile | 0.5-0.6 | * | * | 1.77 - 2.48 [a] | Villa *et al.* (2004) |

| 20 - 40 (60) | Chile | 0.80 | * | * | 1.82 [a] | Villa *et al.* (2008) |
|---|---|---|---|---|---|---|
| 30 - 53 (10) | US | [No supplement]<br>[21mg/day NaF] | *<br>* | *<br>* | 4.4 [a]<br>13.79 [a] | Spencer *et al.* (1970) |

**Table 2.3 B continued**

| Age (years) (N) | Country | Fluoride concentration of water (mg/l) | Levels of Fluoride Ingestion | | | Author |
|---|---|---|---|---|---|---|
| | | | Diet | Toothpaste | TDFI | |
| 39 - 53 (4) | US | [F supplement 9.1 mg or 7.6 mg] | * | * | 4.35 [a] | Spencer *et al.* (1975)[A] |
| 30 - 53 (10) | US | [No supplement]<br>[21mg/day NaF] | *<br>* | *<br>* | 4.4 [a]<br>13.79 [a] | Spencer *et al.* (1970) |

| 20 - 45 | | | | | | |
|---|---|---|---|---|---|---|
| (9) | US | [No supplement] | * | * | 0.41 [a] | Maheshwari *et al.* (1981) |
| (8) | | [5 mg supplement] | * | * | 5.40 [a] | |
| (4) | | [10 mg supplement] | * | * | 10.38 [a] | |

Note:

* - Data not available
[a] - mg/day
[b] – µg/kg bw/day

### 2.2.2. Fluoride Absorption

As mentioned in chapter 1 (section 1.3.1), the absorption of fluoride in the stomach occurs by passive diffusion of hydrogen fluoride (HF) ions (Gharzouli and Senator, 1994), not homeostatically regulated, independent of level of intake (Whitford and Williams, 1986) and dependant on pH (Whitford and Pashley, 1984). The ionic form of fluoride bonds with hydrogen to form HF which diffuses across cell membrane into plasma. This process favours a lower pH, therefore, the higher the acidity of the stomach contents, the more Hydrogen ions are readily available to form HF and hence more rapid fluoride absorption occurs. Whitford and Pashley (1984) found 50% faster fluoride absorption in rats from buffer of pH 2.1 compared to buffer of pH 7.7.

Sharma *et al.* (2010) demonstrated how a low pH environment can ultimately cause tissues to become more sensitive to fluoride and therefore more susceptible to fluoride toxicity in rats. Increased cell stress was observed in rats given 100 ppm fluoridated drinking water in the maturation stage ameloblasts (pH 6.0) compared to secretory stage ameloblasts (pH 7.2) which facilitated the uptake of fluoride and ultimately compromised ameloblast function leading to dental fluorosis.

Considering the importance of pH in the absorption of fluoride from the stomach, Nopakun *et al.* (1989) suggested that such pH dependency can lead to an increase in fluoride absorption and consequently toxicity which can have major implications for the treatment of acute fluoride toxicity as well as in therapeutic applications.

In the proximal small intestine on the other hand, fluoride absorption was not found to be pH dependant within a range of pH 6.0 - 8.0 in rats and occurred as ionic fluoride rather than HF (Nopakun *et al.,* 1990). It was however dependent on concentration as demonstrated by Sato *et al*. (1986) who investigated the absorption of fluoride via the gastro-intestinal tract in rats using the gastrointestinal reflux method. They found a decrease in fluoride absorption following an increase in fluoride concentration when the stomach or small intestine was refluxed for one hour.

The rate of fluoride absorption is almost 100% for NaF and around 80 – 90% of total ingested fluoride from diet and dentifrice is absorbed in the absence of Calcium, Magnesium, Aluminium and other cations which may form insoluble fluoride complexes (Whitford, 1996). NaF is the most absorptive form of fluoride. Sodium monofluorophosphate (MFP) is also absorbed in a similar way to NaF however it requires enzymatic hydrolysis

and occurs at a slower pace than NaF (Buzalaf *et al.*, 2008; Buzalaf and Whitford, 2011). Buzalaf *et al.* (2008) administered a single dose of 2 mgF as NaF or MFP to adult humans aged 19-29 years (n= 4) in fasting conditions. They found fluoride from both NaF and MFP to rapidly absorb, peaking within 20 minutes after ingestion followed by 35% - 41% of the dose being excreted via urine in the 8 hours following ingestion. All in all, concluding that the bioavailability of NaF and MFP was equivalent.

From the perspective of caries prevention, dentrifices containing MFP were found to provide the same level of protection as dentifrices containing NaF amongst children after a 2-year period (n= 2479) (Saporito *et al.,* 2000). However, whilst considering the slower absorption of MFP, dentifrices containing MFP may be an effective and more appropriate alternative for those at risk of fluoride toxicity. Drummond *et al.* (1990) compared five different toothpastes with and without 1000 ppm fluoride in adults (n= 8) in the form of NaF and MFP and found fluoride absorption to be significantly lower from the dicalcium phosphate dihydrate toothpaste which suggests both NaF and MFP are effective forms of fluoride for caries prevention.

Following fluoride absorption and distribution, 35% - 45% of fluoride filtered by the kidneys in rats was also re-absorbed in the proximal tubule by non-ionic diffusion in the form of HF, regardless of urinary pH (Whitford *et al.,* 1976).

### 2.2.3. Fluoride Distribution

Once ingested fluoride has been absorbed, it is rapidly distributed throughout the body distributed via plasma to hard and soft tissue. The main compartments of fluoride distribution within the body are described below.

#### 2.2.3.1. Blood

Fluoride absorbed from the gastro-intestinal tract is distributed via plasma in the blood. Carvalho *et al.* (2006) referred to plasma as the “central compartment” from a pharmacokinetics perspective because it is the fluid from which fluoride passes for distribution and excretion. Fluoride in blood occurs in two forms: ionic and non-ionic, together referred to as ‘total fluoride’ with plasma containing twice as much ionic fluoride than whole blood. It is the ionic form of fluoride which is of most clinical and research significance from a public health, dentistry and medicine perspective (Buzalaf and Whitford, 2011).

Various fasting plasma fluoride concentrations reported in literature have been presented in table 2.4, ranging from 6.0 - 47.0 ng/ml. Cardoso *et al.* (2008) collected plasma and urine samples from five adults aged 27 - 33 years who were receiving a low fluoride diet over a 5-day period. They found plasma fluoride levels of 0.55 ± 0.11 µmol/l (equivalent to 10 ng/ml) to peak at 11am, whilst the lowest plasma fluoride levels were found between 5 and 8 pm of 0.50 ± 0.06 µmol/l (equivalent to 9 ng/ml). They attributed the variations in plasma fluoride levels with transfer to and from bone.

Cardosso *et al.* (2006) determined plasma fluoride concentrations and fluoride intake from diet and dentifrice in 25 - 35-year-old subjects (n= 5) from three different communities (water fluoride concentration = 0.3 ppm, 0.7 ppm and 0.6 - 0.8 ppm). Mean fasting plasma fluoride concentrations of 9.7, 6.8 and 10.5 ng/ml were found for the three communities, respectively.

Sener *et al.* (2007) collected blood plasma from women aged 20 - 30 years who had recently given birth (n= 125). They aimed to determine the fluoride levels in breast milk and plasma of lactating mothers who regularly consumed drinking water with low levels of fluoride (0.3 ppm). Breast milk (5 ml) and blood samples (5 ml) were collected. The levels of fluoride in plasma and breast milk were found to be 6 ng/ml and 17 ng/ml, respectively and significant relation was found between the two.

Maguire *et al.* (2005) investigated the bioavailability of fluoride in drinking water in adults aged 20 - 35 years (n= 20) and found mean baseline fasting plasma fluoride levels of 19.8 ng/ml. Another study investigating fluoride pharmacokinetics in adults aged 24 - 32 years (n= 10) (Whitford *et al.,* 2008) found fasting plasma fluoride concentrations of 17.3 and 20 ng/ml in naturally and artificially fluoridated areas (0.85 ppm), respectively.

Parkins *et al.* (1974) collected fasting blood samples from adult patients aged 17 - 80 years (n= 41) in a hospital setting to compare with fluoride concentrations in bone. Mean fasting plasma fluoride levels of 47.0 ng/ml were detected and were found in equilibrium with bone fluoride.

In children, Ekstrand *et al.* (1983) found mean fasting plasma fluoride concentrations of 15 ng/ml in children aged 3 - 4 years (n= 5) when investigating plasma fluoride concentrations following the ingestion of fluoride tablets and toothpaste. Whereas, Whitford *et al.* (1999) investigated the relation between fluoride concentrations in whole saliva, parotid ductal saliva, and plasma in 5- to 10-year-old children (n= 17) and found mean plasma fluoride

concentrations of 16.9 ng/ml. Data from the US National Health and Nutrition Examination Survey (year 2013-2014) found mean fluoride concentrations of 7 ng/ml in children aged 6 - 11 years and 12 - 19 years (Jain, 2017).

**Table 2.4. Plasma fluoride concentrations in literature**

| Age (years) | Fluoride concentration of water (ppm) | Mean Plasma fluoride level (ng/ml) | Author |
|---|---|---|---|
| 20 - 30 (n= 125) | 0.3 | 6.0 | Sener *et al.,* (2007) |
| 17 - 80 (n= 41) | * | 47.0 | Parkins *et al.* (1974) |
| 25 - 35 (n= 5)<br>25 - 35 (n= 5)<br>25 - 35 (n= 5) | 0.3<br>0.7<br>0.3 | 9.7<br>6.8<br>10.5 | Cardoso *et al.* (2006) |
| 27 - 33 (n= 5) | Low * | 10.0 | Cardoso *et al.* (2008) |
| 21 - 32 (n= 330) | * | 22.0 | Thomas *et al.* (2016) |
| 27 (n= 1) | 0.25 | 10.3 | Ekstrand *et al.* (1977)[A] |
| 24 - 28 (n= 5)<br>27 - 56 (n= 5)<br>10 - 38 (n= 5) | 0.25<br>1.20<br>9.60 | 10.0<br>20.0<br>35.0 | Ekstrand (1978) |
| 27 - 36 (n= 5) | * | 13.3 | Ekstrand *et al.* (1981) |
| 26 - 38 (n= 5) | 0.20 | 9.5 | Oliveby *et al.* (1989)[1] |
| 26 - 38 (n= 5) | 0.20 | 12.4 | Oliveby *et al.* (1989)[2] |
| 26 - 38 (n= 5) | 0.20 | 9.3 | Oliveby *et al.* (1989)[3] |
| 19 - 29 (n= 4)<br>19 - 29 (n= 4) | 0.60 - 0.80<br>0.60 - 0.80 | 21.0<br>22.0 | Buzalaf *et al.* (2008) |
| * | * | 12.7 | Whitford (1996) |
| 24 - 32 (n= 5)<br>24 - 32 (n= 5) | 0.85<br>0.85 | 17.3<br>20.0 | Whitford *et al.* (2008) |
| 20 - 35 (n= 20) | 0.02 | 19.8 | Maguire *et al.* (2005) |

| | | | |
|---|---|---|---|
| 3 - 4 (n= 5) | 1.00 | 15 | Ekstrand *et al.* (1983) |
| 0 - 2 (n= 865)<br>3 - 5 (n= 609)<br>6 - 11 (n= 1280)<br>12 - 19 (n= 1346) | 0.39<br>0.35<br>0.38<br>0.39 | *<br>*<br>7<br>7 | Jain (2017) |
| 5 - 10 (n= 17) | * | 16.9 ng/ml | Whitford *et al.* (1999) |

Note: * Data not available

Peak plasma fluoride concentrations are reached within 30 - 60 minutes following fluoride ingestion, with a half-life of 30 minutes (Whitford *et al.,* 2008; Agali and Shintre, 2016). Once peak plasma levels are reached, plasma fluoride levels then start to decline and correlate with uptake in calcified tissue and renal excretion (Whitford, 1994). Plasma fluoride concentrations return to pre-ingestion levels within 3 - 6 hours (Agali and Shintre, 2016).

Plasma fluoride concentrations can be affected by several factors independent of fluoride dose including site of blood collection, age, haematocrit values which are of greatest influence. (Rugg-Gunn *et al.,* 2011). Other factors include acid-base balance, altitude and genetic background. Plasma fluoride concentrations are also affected by the balance between bone formation and resorption e.g. when fluoride plasma concentrations increase, fluoride is transferred from extracellular fluid to bone and when fluoride plasma concentrations decrease, fluoride is transferred from bone to plasma (Cardoso *et al.*, 2008).

The peak concentration of plasma fluoride is dependent on 1) amount of fluoride ingested; 2) rate of fluoride absorption; 3) volume of fluoride distribution and 4) rate of fluoride clearance from plasma by the kidneys and skeleton (Marya, 2011).

Agali and Shintre (2016) highlighted that although plasma is widely used in dental research, its use in pharmacokinetics models for biomarkers of fluoride exposure is still limited. This indicates the need for further research.

#### 2.2.3.2. Saliva

Fluoride is also distributed via saliva and reflects the absorption levels of fluoride in the body. The three main salivary glands are: 1) parotid; 2) sublingual; and 3) submandibular;

with many minor glands distributed amongst the mucosa and sub-mucosa (throughout the oral cavity). At rest 0.3 ml/minute saliva is produced which can increase to 2.0 ml/minute upon stimulation. The parotid gland is responsible for 20%, submandibular gland for 65% and sublingual / minor glands for 15% of the total saliva produced at rest (Carlson and Ord, 2009). The consistency of secretions produced by each gland vary. Parotid saliva consists of serous cells rich in enzymes and antibiotics. Submandibular saliva consists of serous and mucous secretory cells of an intermediate consistency. Sublingual saliva, on the other hand, predominantly consists of mucous secretory cells with a viscid consistency (Young *et al.*, 2013).

The combination of all these gland secretions as well as gingival crevicular fluid, desquamated oral epithelial cells, microorganisms (and their products) and food debris, all form whole saliva (Hand and Frank, 2014). Fluoride is also returned to the oral cavity systemically via saliva which allows it to work topically. This is of great advantage when bearing in mind that the anti-caries mechanism of fluoride is primarily topical. Fluoride ions interact with hydroxyapatite minerals causing growth of the apatite crystallites which influences remineralisation. The presence of fluoride also leads to the uptake of calcium by calcium-deficient apatite which reverses changes during early dental caries (Ingram *et al.* 2004). In summary, fluoride works by three mechanisms as described by Lynch *et al.* (2004): 1) inhibition of demineralisation, 2) promotion of remineralisation and 3) interference with bacterial growth and metabolism.

Salivary fluoride is also indicative of fluoride excretion levels and it is estimated that only around 1% of fluoride is excreted through saliva (Carlson *et al.,* 1960; Tylenda, 2003).

The concentrations of fluoride found in different types of saliva are summarised in Table 2.5.

**Table 2.5. Fluoride concentrations in different types of saliva. Adapted from Rugg-Gunn et al. (2011)**

| Type of Saliva | Fluoride concentration (ng/ml) | Author |
|---|---|---|
| Whole Saliva | 17 | Yao and Gron (1970) |
| | 13.5 | Oliveby *et al.* (1989) [1, 2, 3] |

| | | |
|---|---|---|
| | 14 - 297 | Fukushima *et al.* (2011) |
| | 41.4 | Twetman *et al.* (1998) |
| Parotid Saliva | 6.5 | Yao and Gron (1970) |
| | 3.23 | Oliveby *et al.* (1989) [1, 2, 3] |
| | 9.8 | Twetman *et al.* (1998) |
| | 9 - 284 | Fukushima *et al.* (2011) |
| Sublingual-Submandibular Saliva | 6.3 | Yao and Gron (1970) |
| | 8.7 | Oliveby *et al.* (1989) [1, 2, 3] |
| | 10.5 | Twetman *et al.* (1998) |

#### 2.2.3.2.1. Whole saliva

Fluoride concentrations in whole saliva are generally higher than ductal saliva. A study by Yao and Gron (1970) found mean fluoride levels in whole saliva to be 17 ng/ml in children and young adults from non-fluoridated communities, refraining from the use of fluoridated dentifrice. Whereas, mean salivary fluoride levels in parotid ductal and sublingual-submandibular saliva from the same individuals were 6.5 and 6.3 ng/ml, respectively.

Salivary fluoride levels are affected by topical fluoride applications e.g. toothpaste, mouthwash, fluoride varnishes, etc. as well as systemic fluoride ingestion via food, drink, etc. (Lynch *et al.,* 2004; Bjornstrom *et al.,* 2004 and Newby *et al.,* 2013). This explains why fluoride concentrations in whole saliva are higher than ductal saliva due to contaminations from food debris and any remaining toothpaste / mouthwash in the mouth, etc. Yao and Gron (1970) also attributed the higher levels of fluoride found in whole saliva to the inclusion of cellular debris and mucus as they found a decrease in fluoride concentration following centrifugation of the whole saliva samples.

A study by Fukushima *et al.* (2011) found whole salivary fluoride levels of 14 ng/ml (in children aged 3 - 7 years) and 297 ng/ml (in adolescents aged 14 - 20 years) from communities consuming 0.09 ppm and 0.72 ppm fluoridated water, respectively.

In other communities with fluoridated water concentrations of 2.31 ppm (high) and 0.36 ppm (low) fluoride, 13-year-old life-long residents were found to have whole salivary fluoride

levels of 47 ng/ml (morning) / 32 ng/ml (noon) and 26 ng/ml (morning) / 17 ng/ml (noon), respectively (Bruun and Thylstrup, 1984). A significant increase in mean salivary fluoride levels was also observed in the same study, 15 minutes after a 30-second mouth rinse with 10 ml local tap water to 92 ng/ml and 32 ng/ml in high and low fluoride communities, respectively. Bruun and Thylstrup (1984) concluded that the increase in whole saliva concentration was most likely due to the direct contact of the oral cavity with fluoride in drinking water and this increased availability of fluoride in the oral fluids is important to reduce caries progression.

Ingle *et al.* (2014) found significantly higher salivary fluoride levels following the use of 1000 ppm fluoride dentifrice in comparison to the use of 500 ppm. They concluded that a reduction in dentifrice fluoride concentration can reduce the risk of dental fluorosis in very young children. However, upon the development of a mature spitting reflex, higher concentrations of fluoride concentrations which increase salivary fluoride levels may outweigh the risk of fluorosis.

#### 2.2.3.2.2. Parotid ductal saliva

Salivary fluoride concentrations, especially from paroid ductal saliva correlate with plasma fluoride concentrations. A study by Whitford *et al.* (1999) investigated the relation between fluoride concentrations in saliva and plasma in 5- to 10-year-old-children (n= 17). Mean fluoride concentrations of 0.72 µmol/l and 0.89 µmol/l were found in parotid ductal saliva and plasma, respectively. They found a significant relation between the two with a proportionality constant of 0.80. Whitford *et al.* (1999) concluded that parotid salivary fluoride concentrations can be used to estimate plasma fluoride concentrations in 5- to 10-year-old children.

On the other hand, in adults, a study by Ekstrand (1977)[A] found a ratio of 0.64 between parotid saliva and plasma fluoride concentrations (Ekstrand, 1977)[A]. Ekstrand (1977)[A] also concluded that salivary fluoride concentrations may be a useful substitute for blood sampling in studies investigating fluoride pharmacokinetics.

A study by Oliveby *et al.* (1989)[3] investigated the influence of salivary flow rate, pH and plasma fluoride concentrations on salivary fluoride excretion and found plasma fluoride to have the greatest influence, regardless of flow rate and pH. Oliveby *et al.* (1989)[3] found close relations between parotid saliva and plasma in 2 separate sub-studies following

ingestion of 1 mg fluoride. In the first study, they found the mean parotid saliva to plasma fluoride ratio to range from 0.29 - 0.65 with a close correlation between the two (r= 0.81). In the second study, they found a mean parotid saliva to plasma fluoride ratio of 0.55 using basal plasma (0.65 µmol/l) and 0.73 when plasma fluoride concentrations ranged from 3.5 µmol/l to 11.6 µmol/l.

#### 2.2.3.2.3. Sublingual-submandibular saliva

Sublingual-submandibular (SL-SM) saliva has a higher correlation with plasma than parotid ductal saliva but concentrations in plasma are still higher with a ratio between SL-SM saliva and plasma of 0.55 ± 0.13 and 0.69 ± 0.11 for unstimulated and stimulated saliva, as demonstrated by Oliveby *et al.* (1989)[1]. A study by Twetman *et al.* (1998) investigated the fluoride levels in whole, parotid and sublingual-submandibular saliva following a 7-day fluoridated milk regimen (1mg F/day) in school children aged 10 - 13 years (n= 12). On day 7, fluoride levels in stimulated and unstimulated whole saliva were 0.40 and 0.44 µmol/l, respectively. In comparison, fluoride levels in parotid saliva and sublingual-submandibular saliva were 0.26 µmol/l and 0.99 µmol/l, respectively. Findings by Twetman *et al.* (1998) support the findings by Oliveby *et al.* (1989)[1] and conclude the sublingual-submandibular glands to be the major contributors of fluoride in the oral cavity.

With respect to all three saliva types discussed, Buzalaf (2011) stated both parotid and sublingual-submandibular ductal saliva as the preferred markers of plasma fluoride concentrations with the latter having greater preference.

### 2.2.3.3. Mineralised Tissue

In total, around 99% of fluoride absorbed into the body is associated with calcified tissue (Whitford, 1994; NRC, 2007) including bone, enamel, dentin and nail. Different types of mineralised tissue contain varying amounts of fluoride due to their varying structure. Bone and dentin have similar amounts of fluoride. Fluoride in enamel on the other hand is lower compared to bone and dentin (Whitford, 1994). High fluoride concentrations are known to increase cancellous bone density which can increase the risk of fluorosis. Adult patients with chronic intestinal failure (n= 31) receiving home parenteral nutrition with a daily fluoride intake of 3.1 mg/day for women and 3.8 mg/day for men were found to have increased

spinal bone mineral density, of which two patients also displayed symptoms of fluorosis (Bouletreau *et al.,* 2006).

Chachra *et al.* (2008) explained the multiple ways in which fluoride interacts with mineralised tissue. They explained how low doses can passively incorporate into mineralised tissue, stabilising it against dissolution; higher doses can alter the amount and structure of tissue; and very high doses are associated with weakening of the skeleton and mottled and discoloured teeth.

Fluoride in calcified issue is reversibly bound which allows some to be released during bone remodelling. The concentrations in bone and dentin are proportional to level of intake which means they increase with age due to the increased uptake of fluoride over time (Ishiguro *et al.,* 1993; Larsen *et al.*, 1985). Ishiguro *et al.* (1993) found the pattern of fluoride distribution in the rib to increase with age in adults aged 20 - 93 years (n= 110) with a significant gender difference at the age of 55 years. Larsen *et al.* (1985), on the other hand found the prevalence of enamel lesions in the enamel stage of late secretion / early maturation of tooth development in children previously participating in a fluoride tablet program (0.5 mgF/day) (n= 70) as oppose to those without the programme (n= 40).

Unlike bone and dentin, fluoride in enamel decreases with age. Fluoride in bone and dentin reflect the level of intake, whereas, fluoride in enamel reflects exposure during tooth development. Excess fluoride uptake into mineralised tissue can become problematic increasing the risk of dental and skeletal fluorosis (Everett, 2011).

#### 2.2.3.4. Soft Tissue

Soft tissue contains less than 1% of fluoride in the body (Zohoori and Duckworth, 2017). Fluoride concentrations in extracellular fluid and plasma are higher than intracellular fluid due to the high pH of extracellular fluid. Whitford *et al.* (1979) investigated the short-term distribution of fluoride in 12 different soft tissue in rats. The rats were administered fluoride (18F) intravenously and killed at 5, 10, 15, 20, 30, and 60 minutes following the dose. They found none of the soft tissues to strongly bind to fluoride, 18F. Ratios of tissue water to plasma from their findings lead to the idea that fluoride distribution in soft tissue is determined by the diffusion equilibrium of HF.

pH levels in soft tissue are not always stable and can change leading to a flux of fluoride ions into and out of cells (Buzalaf and Whitford, 2011). The amount of fluoride distributed to soft tissue is also affected by the uptake of fluoride in bone. A study by Inkielewicz and Krechniak (2003) administered male rats (n= 8) with 0.3 mg/l NaF drinking water for the control condition and 5 mg/l and 25 mg/l NaF drinking water for the experimental condition for 12 weeks. They measured urinary fluoride excretion weekly and the fluoride content of the liver, kidney, brain, testis, and serum, before exposure as well as 2, 4 and 12 weeks following exposure. Inkielewicz and Krechniak (2003) found a dose-dependent increase in urinary fluoride and a dose- and time-dependant increase in the fluoride content of the tissues and organs measured. They attributed the increase in fluoride levels over time to the balance in absorption, storage in bones, and excretion being achieved. Along with pH and uptake in bone, fluoride distribution to soft tissue is also dependant on the velocity of blood flow to the various tissues and organs (Whitford *et al.,* 1979; Preedy, 2015).

### 2.2.4. Fluoride Excretion

The two main routes of fluoride excretion from the body are renal and faecal excretion. Fluoride from plasma is distributed throughout the body and then cleared by the kidneys to be excreted via urine. Almost 10% of ingested fluoride is not absorbed by the gastrointestinal tract and consequently excreted via faeces (Villa *et al.*, 2000). Of the fluoride ingested, in children around 35% is excreted via urine (Villa *et al.,* 2000), whereas in adults, around 50% of the total daily fluoride ingested is excreted via urine (Spencer *et al.* 1970). Faecal fluoride excretion, on the other hand was found to be very low, with a mean excretion of 0.88 mg/day with and 0.29 mg/day without the intake of 21 mg NaF a day (Spencer *et al.* 1970).

Fluoride is also excreted via sweat. However, the amount of fluoride excreted through sweat is minimal. The amount of sweat produced by an individual is dependent on the gender, health, living and work conditions and climate of an individual (Ingram and Mount, 2012). Zohoori and Duckworth (2017) suggested that individuals living in hot climates or during heavy / prolonged exercise, may excrete a significant higher amount of fluoride.

Table 2.6 provides an overview of the fluoride concentrations found in sweat, summarised from Rugg-Gunn *et al.* (2011).

**Table 2.6. Fluoride concentrations in sweat.**

| Sweat Fluoride concentration (mg/l) | Author |
|---|---|
| 0.3 - 1.8 | McClure *et al.* (1945) |
| 0.3 - 0.9 | Crosby and Shepherd (1957) |
| 0.05 | Murray *et al.* (1991) |
| 0.019 - 0.057 | Whitford (1996) |

This section primarily focuses on urinary fluoride excretion in children and adults.

#### 2.2.4.1. Urinary Excretion in children

As mentioned above, the fraction of total daily fluoride ingested that is excreted (FUFE) was found to be 35.5% or 0.358 mg/day in children aged 3 - 5 years (n= 20) (Villa *et al.,* 2000). This is supported by the findings of Villa *et al.* (1999), who found urinary fluoride excretion (UFE) of 0.229 mg/day in 3-5-year-old children (n= 88) living in areas of 0.57-0.62 mgF/l and 0.526 mg/day for those children receiving 1mg fluoride supplement in 50 ml orange juice.

Rate of UFE is calculated by multiplying the 24-hour urinary volume by urinary fluoride concentration (Zohoori *et al.,* 2015). The range of UFE in children is around 0.17 to > 0.60 mg/24h following fluoride intake of around 0.40 to > 1.63 mg/24h. In adults the range of UFE is 1.00 to > 3.00 mg/24h following fluoride intake of around 1.31 to > 5.00 mg/24h (Rugg-Gunn *et al.* 2011). However, a definitive range of fluoride excretion cannot be established because of external factors on urinary excretion as well as fluoride exposure (Rugg-Gunn *et al.* 2011). The factors that affect UFE include urinary pH, composition of diet, altitude, glomerular filtration rate and overall kidney functioning (Mehta, 2013; Martinez-Mier, 2011; Schiffl and Binswanger, 1980; Gizewska and Machoy, 1988 and Collins, 2016).

After being filtered from the glomerulus, around 10 - 90% of the fluoride which enters the renal tubules is reabsorbed whilst the rest is excreted. The mechanism for renal reabsorption is similar to that of gastric absorption and occurs by passive diffusion of HF, again dependant on a low pH to cross the tubular epithelium (Buzalaf and Whitford, 2011).

The relationship between 1) total daily F intake (TDFI) and daily urinary F excretion (DUFE), and 2) TDFI and fractional urinary F excretion (FUFE) was investigated in children aged 6 - 7 years from low fluoridated (0.30 mgF/l) (n= 21) and naturally fluoridated (1.06 mgF/l) (n= 12) areas in north-east England (Zohoori *et al.* 2013). Twenty-four-hour urine samples were obtained. FUFE was calculated using the ratio between DUFE and TDFI. Mean (SD) TDFI was found to be 0.038 (0.027) mg/kg per day for low fluoridated areas and 0.076 (0.038) mg/kg per day for naturally fluoridated areas. Mean (SD) DUFE was 0.012 (0.006) mg/kg per day for the low fluoridated areas and 0.017 (0.007) mg/kg per day for the naturally fluoridated areas. Zohoori *et al.* (2013) found no correlation between TDFI and DUFE but did find a statistically significant negative correlation between FUFE and TDFI.

Maguire *et al.* (2007) measured total fluoride intake, UFE and estimated fluoride retention in children aged 6 - 7 years, living in either sub-optimally fluoridated (0.47 mgF/l) (n= 9), optimally fluoridated water (0.82 mgF/l) (n= 6) or non-fluoridated (0.08 mgF/l) (n= 18) areas, as discussed in section 2.2.1. They collected 24-hour urine samples to measure UFE and urine volume. Urine data was used to estimate fractional urinary fluoride excretion (FUFE) and fluoride retention. Maguire *et al.* (2007) found mean FUFE to range from 32% (±13%) for the optimally fluoridated area to 44% (±33%) for the low-fluoride area. No correlation was found between fluoride retention and the fluoride concentration of home water supply, but a strong positive correlation was found between fluoride retention and total daily fluoride intake. These findings support Zohoori *et al.* (2013) who also found fluoride retention to correlate with fluoride intake. A difference of +0.087 has been found between ingestion and urinary excretion, equivalent to 80% excretion (Zohouri and Rugg-Gunn, 2000).

Ketley and Lennon (2000) investigated fluoride excretion under various conditions of fluoride intake and estimated the fractional urinary excretion of fluoride in individual 4 to 5-year-old children (n= 8) children participating in a school milk fluoridation scheme. They collected individual urine samples for a continuous period of 55 hours and found a mean daily urinary fluoride excretion to be 0.33 mg/day, under usual conditions of fluoride intake (i.e. milk containing 0.5 mg fluoride, customary diet and tooth brushing with fluoride toothpaste).

Mean UFE values for children receiving fluoridated milk (0.5 mg) in non-fluoridated areas (<0.1 mg/l) i.e. 0.30 mg were found between the values reported for children not receiving fluoridated milk, when comparing with children not receiving fluoridated milk living in non-

fluoridated (<0.1 mg/l) i.e. 0.21 mg and fluoridated areas (0.8 - 1.0 mg/l) i.e. 0.36 mg (Ketley *et al.,* 2002).

#### 2.2.4.2. Urinary excretion in adults

In adolescents (aged 11 - 14 years (n= 19)) and adults (aged 19 - 75 years (n= 73)) living in fluoridated areas in Chile (0.6 mgF/l), FUFE values have been reported as 0.35 and 0.75, respectively (Villa *et al.,* 2004). Mean levels of FUFE in female adults, aged 20 - 40 years (n= 60) have found to be 0.69 (Villa *et al.,* 2008) which supports the findings from Ville *et al.* (2004).

In adults, 50% of ingested fluoride was found to be excreted via urine, with and without the intake of 21 mg NaF a day (Spencer *et al*. 1970). Whilst Maheshwari *et al.* (1981) reported approximately 90% of the excreted fluoride to be found in urine. In adults, excess fluoride excretions were also measured following the provision of fluoride supplements containing either 9.1 mg fluoride as NaF (ingested daily for 32 days) or 7.6 mg fluoride as fish protein concentrate (FPC) (ingested daily for 26 days). Excess fluoride was excreted primarily via urine (87%) and little amounts via stool (13%) (Maheshwari *et al.* (1981)). Excess fluoride excretions were found to be low and short ranging from 6 - 12 days following discontinuation of the fluoride supplements (Spencer *et al*. 1975)[A]. These findings suggest that the recent intake of fluoride may be excreted from the body after a short period of time. However, many factors can affect the rates of retention and hence excretion.

Studies on Urinary fluoride excretion in children and adults have been summarised in table 2.7 A and 2.7 B, respectively.

**Table 2.7. Studies on Urinary Fluoride Excretion (UFE) levels.**

**A. Studies on Urinary Fluoride Excretion (UFE) levels in children**

| Age (years) (N) | Country | Water fluoride (mg/l) | Fluoride Source | Method of urine collection | UFE | | Author |
|---|---|---|---|---|---|---|---|
| | | | | | Mg/day | Mg/kg bw | |
| 1 - 3 (7) | UK | 0.8 | * | Spot sample | 0.33 | * | Zohouri *et al*. (2006) |
| 6 - 7<br>(21)<br>(12) | UK | 0.30<br>1.01 | Tap water, usual diet and dentifrice | 24-hour collection | *<br>* | 0.012<br>0.017 | Zohouri *et al*. (2013) |
| 6 - 7<br>(18)<br>(8) | UK | 0.08<br>0.47 | Tap water and usual diet | 24-hour collection | 0.203<br>0.239<br>0.323 | *<br>*<br>* | Maguire *et al.* (2007) |

| (5) | | 0.82 | | | | | |
|---|---|---|---|---|---|---|---|

**Table 2.7 A continued**

| Age (years) (N) | Country | Water fluoride (mg/l) | Fluoride Source | Method of urine collection | UFE | | Author |
|---|---|---|---|---|---|---|---|
| | | | | | Mg/day | Mg/kg bw | |
| 1.8 - 5.2<br>(19)<br>(22)<br>(16) | Ireland<br>UK | 0.8 - 1<br><0.1<br><0.1 | *<br>*<br>Milk 0.5 mg | 24-hour collection | 0.36<br>0.21<br>0.30 | *<br>*<br>* | Ketley *et al.* (2002) |
| 4 - 5 (8) | UK | <0.1 | Milk 0.5 mg | 55-hour collection | 0.33 | 0.017 | Ketley and Lennon (2000) |
| 4<br>(44) | UK | 0.8 - 1.1 | * | 24-hour collection | 0.42 | * | Rugg Gunn *et al.* (1993) |

| (53) | Sri Lanka | | * | | 0.55 | * | |
|---|---|---|---|---|---|---|---|
| 4 (78) | Iran | 0.33 | * | 24-hour collection | 0.339 | 0.024 | Zohouri and Rugg Gunn (2000) |
| <4<br>(14)<br>(15) | Brazil | 0.6-0.8<br><0.3 | *<br>* | 24-hour collection (and faeces) | *<br>* | 0.026-0.039<br>0.005-0.008 | Zohoori *et al*. (2013) |

**Table 2.7 A continued**

| Age (years) (N) | Country | Water fluoride (mg/l) | Fluoride Source | Method of urine collection | UFE | | Author |
|---|---|---|---|---|---|---|---|
| | | | | | Mg/day | Mg/kg bw | |
| 3 - 5 (42)<br>(46) | Chile | 0.57 - 0.62<br>0.57 - 0.62 | * | 24-hour collection | 0.229<br>0.526 | 0.015<br>0.028 | Villa *et al*. (1999) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | F supplement (1 mg/50 ml juice) | | | | |
| 3 - 5 (20) | Chile | 0.5 - 0.6 | * | 24-hour collection | 0.358 | 0.022 | Villa *et al.* (2000) |
| 0.19 - 0.54 (5)<br>0.15 - 0.31 (5) | Sweden | 1.0 | Breast milk (6µg/F)<br>Formula milk (891 to 1,012 µg/F) | 24-hour collection (and faeces) | 0.030<br>0.360 | *<br>* | Ekstrand *et al.* (1984) |

Note:

* - Data not available.

**B. Studies on Urinary Fluoride Excretion (UFE) levels in adults.**

| | Country | | Fluoride Source | | UFE | Author |
|---|---|---|---|---|---|---|

| Age (N) | | Water fluoride (mg/l) | | Method of urine collection | Mg/day | Mg / kg bw | |
|---|---|---|---|---|---|---|---|
| 18 - 75 (115) | Chile | * | * | 24-hour collection | 0.49 - 2.08 | * | Villa *et al.* 2010 |
| 19 - 73 (73) | Chile | 0.5 - 0.6 | * | 24-hour collection | 1.32 - 1.68 | * | Villa *et al.* (2004) |
| 20 - 40 (60) | Chile | 0.80 | * | 24-hour collection | 1.24 | * | Villa *et al.* (2008) |
| 30 - 53 (10) | US | * | 21 mg/day supplement | 24-hour collection | 7.52 | * | Spencer *et al.* (1970) |
| 39 - 53 (4) | US | * | 9.1 mg or 7.6 mg supplement | 24-hour collection | 2.20 | * | Spencer *et al.* (1975)[A] |

**Table 2.7 B continued**

| Age (N) | Country | Water fluoride (mg/l) | Fluoride Source | Method of urine collection | UFE | | Author |
|---|---|---|---|---|---|---|---|
| | | | | | Mg/day | Mg / kg bw | |
| 20 - 45 | | | | | | | |
| (9) | US | * | No supplement | 24-hour collection | 0.75 | * | Maheshwari *et al.* (1981) |
| (8) | | * | 5 mg supplement | | 3.57 | * | |
| (4) | | * | 10mg supplement | | 6.76 | * | |

Note:

* - Data not available

### 2.2.5. Fluoride Retention

Following distribution, some of the fluoride which is not excreted is retained in the body. Fluoride retention can be estimated by measuring the fluoride balance in the body which is the difference between the systemic fluoride uptake and excretion. A positive balance which indicates the retention of fluoride in the body is observed when total fluoride intake is greater than total fluoride excretion. A negative balance, oppositely, which indicates loss of fluoride from the body is observed when total fluoride excretion is greater than total fluoride intake (Maguire and Zohoori, 2013).

Spencer *et al.* (1970) measured fluoride balances in adults before and during the intake of NaF, and after its discontinuation whilst maintaining controlled dietary conditions. They found positive fluoride balances averaging +1.9 mg/day without NaF and +5.4 mg/day with the addition of 9.1 mg/day NaF. Fluoride retention was found to correspond to 42.9% of the fluoride intake without NaF and 39.3% with NaF. However, upon discontinuing NaF intake in participants, the fluoride balance became lower than that observed without NaF.

Spencer *et al.* (1975)[B] found high retention of fluoride during high intake of supplemented doses of 10, 20 or 45 mg NaF / day over the course of several weeks in osteoporosis patients aged 48 - 77 years (n= 7) but only up until supplementation was discontinued. The fluoride balance rapidly returned to control levels following discontinuation of high fluoride intake. These findings support Spencer *et al.* (1970) and indicate that recent fluoride intake primarily impacts fluoride retention in the short term.

The majority of retained fluoride has been associated with hard tissue. The mechanism by which fluoride is incorporated into bone starts by a net transfer of fluoride from plasma to the hydration shells of bone crystallites when plasma concentrations raise and vice versa when concentrations fall (Buzalaf *et al.,* 2005). This is followed by incorporation of fluoride into precursors of hydroxyfluorapatite and eventually apatitic lattice (Buzalaf and Whitford, 2011). Incorporation of fluoride into the bone can affect bone strength and to determine the relation between bone fluoride content and bone mass / skeletal fragility, Einhorn *et al.* (1992) treated 21-day-old rats with four different doses of fluoride as NaF. They found over 50% turn over in the skeleton of the rats treated with NaF with a linear increase in the bone fluoride concentration in relation to the four different doses, however, no changes in the mechanical properties of bone were observed.

Villa *et al.* (2010) described how fluoride retention correlates with the developing skeleton which in turn correlates with age. The infant skeleton retains higher in fluoride due to its continuing development. A review of existing data by Villa *et al.* (2010) found estimated fractional fluoride retention values of 0.55 (55%) and 0.36 (36%) for children aged <7 years (n= 212) and adults aged 18 - 75 years (n= 283) with a total daily fluoride intake of 0.5 mg and 2 mg, respectively. These findings indicated the greater retention of fluoride in the growing skeleton of young children.

In another study, Zohoori *et al.* (2013) found 61% of ingested fluoride to be retained within the body of 4-year-old children from fluoridated (0.64 mg/l) (n= 15) and non-fluoridated (0.04 mg/l) (n= 14) areas of Brazil. This value is close to the retention value of 55% by Villa *et al.* (2010). A significant correlation with total daily fluoride intake was also found by Zohoori *et al.* (2013). They also investigated the relation between fluoride retention and age / BMI but found no significant correlations.

Guo *et al*. (1988) also found 60% of fluoride to be redeposited in the skeleton of growing rats during bone resorption. They preloaded the rat skeleton with fluoride using highly fluoridated water i.e. 50 mg/l along with labelling it with [3H] tetracycline (3H-TC) to measure bone turnover. They then provided the rats with low fluoride intake and compared the loss of bone fluoride undergoing normal bone turnover or turnover accelerated by a low calcium (Ca) intake. They found the re-deposition of fluoride to correlate with mineral deposition, therefore attributing the retention of fluoride predominantly to re-deposition of reabsorbed fluoride instead of passive retention linked to low bone turnover.

Fluoride retention in soft tissue on the other hand is minimal with slightly higher retention rates reported in the kidneys compared to other soft tissue. This occurs due to the renal re-absorption of fluoride. Ekstrand *et al*. (1980) administered 5 healthy adults with 3 mg NaF tablets on two occasions: 1) with $NH_4Cl$ to induce the production of acidic urine and 2) with $NaHCO_3$ to induce the production of alkaline urine. They found lower urinary fluoride excretion as well as longer plasma fluoride half-life for acidic urine compared to alkaline. They suggested that the decreased renal clearance during acidic conditions was caused by the increased reabsorption of non-ionic HF.

Fluoride is also retained in oral soft tissue. Zero *et al.* (1992) found a strong correlation between the fluoride concentration of whole saliva and specific soft tissue sites in the mouth measured over a one-hour period, following the use of a placebo dentifrice (0.4 ppm F), fluoride dentifrice (1100 ppm F), fluoride rinse (226 ppm F), or fluoride gel (5000 ppm F).

The highest levels of fluoride retention were found in the tongue and lower posterior vestibule followed by the upper posterior buccal vestibule and upper anterior labial vestibule. The lowest levels of fluoride were found to be retained by the lower anterior vestibule and the floor of the mouth. Fluoride retention can be affected by renal functioning and remodelling rates along with the genetic background, age and gender of the subject (Pessan and Buzalaf, 2011).

Infants living in areas of 1 ppm fluoride, receiving formula milk (bottle fed) (n= 5) (with daily fluoride dose of 891 to 1,012 μg) have been found to retain more than 50% of the ingested fluoride dose compared to infants which are breast fed (n= 5) (with daily fluoride dose of 6 μg) (Ekstrand *et al.,* 1984). It has previously been suggested that the rapidly growing skeleton of infants plays a role in increasing fluoride retention. However, Ekstrand *et al.* (1984) also suggested that a slow elimination rate for fluoride due to the underdeveloped renal handling capacity of infants may also contribute to the increased retention of fluoride.

In adults, excess fluoride excretions were measured following the provision of fluoride supplements containing either 9.1 mg fluoride as NaF (ingested daily for 32 days) or 7.6 mg fluoride as fish protein concentrate (FPC) (ingested daily for 26 days), as discussed in section 2.2.4. Excess fluoride excretions were found to correspond to 9.1% of the retained fluoride given as NaF and to 14.1% of the retained fluoride given as FPC (Spencer *et al.* 1975)[A].

### 2.2.6. Factors affecting Fluoride metabolism

Fluoride metabolism has been suggested to be affected by several factors including genetics, altitude, nutritional status and exercise (Buzalaf, 2011). There is limited evidence relating these factors. In relation to genetic predisposition to dental fluorosis, some populations have shown to present an increased susceptibility to dental fluorosis compared to others (Everett *et al.,* 2002; Preedy, 2015). In mice, the A/J mouse strain has been found to be highly susceptible to dental fluorosis after examination of 12 inbred strains of mice (Everett *et al.,* 2002). Susceptibility to dental fluorosis is known to occur through MAPK signaling pathways which can alter gene expression, induce cell stress or cell death (Everett, 2011). Karube *et al.* (2009) found NaF-induced apoptosis (cell death) in MDPC-23 odontoblast-like cells after treatment with 5mM NaF for 24 hours.

Altitude, on the other hand, also affects fluoride metabolism. People living at higher altitudes are known to have greater fluoride retention. A study performed with 284 Tanzanian children aged 9 - 19 years living at 3 different altitudes (100 metre (m), 840 m and 1463 m) were examined for dental fluorosis and caries. Severe enamel disturbances were found in those living at the highest altitude despite the negligible water fluoride concentrations in that area (Yoder *et al.,* 1998). The cause of this may be due to the hypoxia at high altitudes which decreases urinary pH, reducing fluoride renal clearance leading to increased fluoride retention (Buzalaf and Whitford, 2011).

Acid-Base disturbances in general, often induced by diet also affect fluoride metabolism due to the pH dependency of renal excretion / re-absorption (Buzalaf *et al.*, 2015; Ekstrand *et al.*, 1980). Whilst considering the importance of the renal system in fluoride metabolism, any renal impairment which affects the renal functioning can also affect the metabolism of fluoride. It is thought that tubular reabsorption would diminish due to expansion of the extracellular fluid compartment (Schiffl and Binswanger, 1980). Such renal impairment would lead to greater retention of fluoride (Spencer *et al.,* 1980). Two-fold to three-fold increases have been seen in patients with chronic renal failure, receiving 40 to 60 mgF/day. This has been accompanied with slight increases in urinary fluoride excretion and heavy tissue absorption (Parsons *et al.*, 1975).

Exercise has also been suggested to have an effect on the metabolism of fluoride. The decrease in blood flow and the increase in lactic acid concentration in the blood during moderate and vigorous exercise can reduce glomerular filtration and urinary pH. These physiological adaptations may reduce fluoride excretion and increase renal reabsorption (Freund *et al.*, 1991) or possibly prolonged sessions of exercise could increase fluoride absorption in more irrigated tissues (Buzalaf, 2011). However, the effects of exercise on fluoride metabolism are not fully understood. The association between exercise and fluoride metabolism will be discussed later in section 2.6.

## 2.3. Biological samples: collection methods

Several methods have been developed over time for the collection of biological samples to facilitate fluoride pharmacokinetic studies. The methods used to collect blood, saliva and urine have been discussed below.

### 2.3.1. Blood

The current method for blood collection of samples for studying fluoride pharmacokinetics primarily involves venous whole blood sampling to gain plasma or serum. There are various types of equipment used to collect blood samples. The most traditional method is the needle and syringe method. However, the safest method is the Vacutainer™ system, which can be attached to either a multi-sampling needle or a butterfly needle (catheter) and luer adaptor (Nutbeam and Daniels, 2009). Although, it is the safest method, the loading of blood collection tubes onto the Vacutainer™ requires great dexterity and practice to ensure the needle stays in place (Nutbeam and Daniels, 2009). In cases where repeated blood samples are required at short time intervals, a cannula can be used to collect blood samples. This prevents the need for repeated venepuncture, however, the main limitation arises from clotting which can occur in the cannula (Eston and Reilly, 2013).

Blood collection tubes used for the collection of plasma contain anti-coagulants such as EDTA, heparin or sodium citrate and it is important to ensure the volume of blood filling the tubes are adequate to maintain the optimum blood/additive ratio (Tuck *et al.,* 2009). Blood collection tubes used for the collection of serum are known as serum separator tubes. Serum separator tubes contain a gel barrier that separates the fluid portion from the solid portion of blood which is then allowed to clot. Clotting takes around 30 minutes and then the sample is required to be centrifuged for 15 minutes to gain serum (DiPiro *et al.,* 2010).

Although blood plasma is an accurate measure of fluoride absorption levels, the collection of blood through venous sampling can be invasive, unethical and poses the risk of harm, especially when involving young children. Guidelines by the Royal College of Paediatrics, Child Health: Ethics Advisory Committee (2000) have stated that *"Where children are unable to give consent, by reason of insufficient maturity or understanding, their parents or guardians may consent to the taking of blood for non-therapeutic purposes, provided that they have been given and understand a full explanation of the reasons for blood sampling and have balanced its risk to their child"*. They also add that *"We believe that this has to be the child's decision. We believe that it is completely inappropriate to insist on the taking of blood for non-therapeutic reasons if a child indicates either significant unwillingness before the start or significant stress during the procedure*".

Obtaining consent for the collection of venous blood can prove to be challenging, even in adults and is often not readily obtained. Alternatively, capillary blood sampling which is less

invasive compared to venepuncture has also been used for fluoride analysis (Ekstrand, 1977)[A]. However, it is not practical to obtain plasma from capillary whole blood due to the extremely small volume of sample collected. Moreover, the site of blood collection can affect the fluoride concentration in plasma (Buzalaf, 2011; Whitford, 1996). An advantage of collecting plasma from venous blood for fluoride analysis arises from the storage of such samples. It is recommended that plasma can be stored for up to one year at -18 ºC or for seven years at -65 ºC. Upon thawing it can be kept for a further five days at 1 - 6 ºC (Hess, 2010).

A study by Cardosso *et al.* (2008) which investigated daily variations in human plasma fluoride concentrations amongst adults aged 27 - 33 years (n= 5) collected blood samples at five-time points during the day: 8am, 11am, 2pm, 5pm and 8pm. Around 16 ml of blood was collected at each time point, of which 8 ml was transferred to heparinized plastic tubes (15 µl heparin) with a fluoride content of 9.678 µmol/l and 8 ml was transferred to plastic tubes with no additives.

A similar method was previously used by Cardosso *et al.* (2006) to determine plasma fluoride concentrations and fluoride intake from diet and dentifrice in 25 - 35-year-old subjects (n= 5). They collected 5 ml blood samples at 8am, 12pm, 4pm and 8pm. Blood samples were transferred to heparinized plastic tubes (15 µl heparin) with a fluoride content of 9.678 µmol/l. Cardosso *et al.* (2006) stated the contribution of fluoride from heparin was negligible, totalling around 2.76 ng to each 5 ml blood sample.

The use of heparinized polyethylene tubes has also been demonstrated by Sener *et al.* (2007) who collected blood plasma from women aged 20 - 30 years who had recently given birth (n= 125). Blood samples (5 ml) were centrifuged in fluoride-free heparinized polyethylene tubes and stored at -18ºC till further analysis using a fluoride ion-selective electrode combined with an ion-analyser.

Maguire *et al.* (2005) investigated the bioavailability of fluoride in drinking water in adults aged 20 - 35 years (n= 20) by collecting 5 ml blood samples as part of their study using an intravenous catheter at the following time points: before (fasting) and at 15, 30, 45, 60, 90, 120, 150, 180, 240, 360 and 480 minutes following ingestion of drinking water samples. The blood samples were drawn into sodium heparinate-sprayed fluoride-free plastic tubes and centrifuged at 1300 rpm for 5 minutes to gain plasma which was stored in a freezer at -20°C until fluoride analysis.

A similar method was used by Whitford *et al.* (2008) who collected 5 - 6 ml blood samples at several time points i.e. before and 20, 40, 60, 90, 120, 180, 240, 300 and 360 minutes post fluoride ingestion (water containing doses of 0.67 and 5.45 mg/l) in a study investigating fluoride pharmacokinetics in adults aged 24 - 32 years (n= 10). They also collected venous blood samples into lightly heparinised syringes to gain plasma samples which were then analysed using the ion specific electrode.

A study mentioned previously by Parkins *et al.* (1974) used a buffer i.e. 1mM trisodium citrate (ratio 1:1) to adjust the pH and ionic strength of the plasma sample gained from adult patients aged 17 - 80 years (n= 41) in a hospital setting to compare with fluoride concentrations in bone. The plasma sample was then analysed using the fluoride ion-selective electrode.

Although, the majority of studies relating fluoride pharmacokinetics have collected blood samples from adults, a few studies have reported blood collection from children. The lack of studies using blood collection in children is due to the ethical implications surrounding venous blood sampling. Ekstrand *et al.* (1980) have also demonstrated the use of capillary and venous blood sampling amongst four children (aged 4, 5, 12 and 14 years) during an investigation of plasma fluoride concentrations and urinary fluoride excretion following the use of a fluoride varnish – Duraphat. In phase one of the study, capillary blood samples (1 ml) were collected in fluoride-free heparinised (Vitrum) polyethylene micro-tubes at an hourly rate for 8 hours following varnish application. In phase two, venous blood samples were collected every 15 minutes for the first hour followed by capillary blood samples at 5, 8 and 24 hours post varnish application. Ekstrand *et al.* (1980) found the highest plasma fluoride concentrations from the study to range from 60 - 120 ng/ml and classified them as ‘below toxic’, suggesting the side effects of fluoride should be minimal following fluoride varnish application.

Kumar *et al.* (2017) collected blood samples from children as part of their study to determine the relation between water, urine, serum fluoride and fluorosis in Indian school children aged 8 - 15 years (n= 66). They collected 5 ml venous blood samples using sterilized needles and syringes. Blood samples were transferred to a dried glass vial at room temperature for 20 minutes followed by being centrifuged at 2000 rpm for 10 minutes at 4°C to gain serum samples which were stored at -20°C until fluoride analysis using the Orion fluoride ion-specific electrode. They found mean serum fluoride levels of 0.15, 0.34 and

0.17 mg/l from children living in areas of 2.17, 2.18 and 2.22 mg/l fluoride, respectively and a significant correlation between fluoride levels in water, urine and serum and fluorosis.

Singh *et al.* (2014) evaluated the effect of chronic excess fluoride intake on thyroid function among Indian school children aged 8 - 15 years from endemic (n= 60) and non-endemic fluorosis (n= 10) areas. They collected blood samples in plastic bottles and left them to clot at room temperature followed by centrifugation to gain serum. The serum samples were analysed for fluoride potentiometrically using an ion 85 ion analyzer and a fluoride ion specific electrode. They found fluoride levels in blood serum to range from 0.02 - 0.77 mg/l and have significant relations with thyroid hormone (FT3/FT4) and TSH concentrations. A significant relation was also found between water fluoride to urine and serum fluoride concentrations.

### 2.3.2. Saliva

Fluoride concentrations in parotid ductal saliva have been reported to correlate well with plasma fluoride concentrations. Therefore, ductal saliva which can be collected in a non-invasive manner is deemed to be an appropriate biomarker for fluoride exposure (Fukushima *et al.*, 2011). Whitford *et al.* (1999) collected parotid saliva using a Lashley cup from children aged 5 - 10 years (n= 17) and found parotid fluoride concentrations to significantly relate to plasma fluoride concentrations ($p < 0.0001$) by a proportionality constant of 0.80. In adults, on the other hand, the ratio between SL-SM saliva and plasma was found to be 0.55 ± 0.13 and 0.69 ± 0.11 (n= 5), as mentioned previously (section: 2.2.3) for unstimulated and stimulated saliva, respectively (Oliveby *et al.* 1989)[1].

The three main types of saliva used in studies of fluoride pharmacokinetics are whole saliva, parotid ductal saliva and sublingual-submandibular (SL-SM) saliva, as discussed in section 2.2.3.

#### 2.3.2.1. Whole saliva

Whole saliva, in general, can be collected non-invasively with ease and without the need for trained personnel or specialised skills. Consent is often easily gained for the collection of whole saliva. Methods most commonly used for saliva collection include draining, spitting, suction and swabbing (Topkas *et al.,* 2012). Topkas *et al.* (2012) investigated commercially available saliva collection methods. They collected saliva from healthy adult participants (n

= 17) aged 18 - 33 years. Unstimulated saliva was collected by 1) passive drooling into a sterile specimen container and 2) by placing the commercially available Salimetrics® Oral Swab (SOS) under the tongue for 2 minutes. Saliva was then collected from the SOS by centrifugation at 2095 rcf for 15 minutes at 22°C.

Stimulated saliva was collected using 1) commercially available Salivette® (Sarstedt) Cotton and Synthetic collection device, the swabs were placed in the participants mouth and they were asked to chew on the swab for 45 seconds to stimulate saliva. Saliva was gained by centrifugation at 233 rcf for 2 minutes at 22°C. Stimulated saliva was also collected using 2) commercially available Greiner Bio-One Saliva collection system®, participants firstly rinsed their mouths with a rinse solution consisting of 27 mM citrate buffer followed by rinsing with a saliva stimulant consisting of 39 mM citrate buffer and tartrazine for 2 minutes. The saliva was collected in a sterile specimen container.

Topkas *et al.* (2012) found significant differences in the salivary flow rates between methods used to collect stimulated and unstimulated saliva. The unstimulated methods i.e. drool method and SOS yielded salivary flow rates of 0.60 ± 0.39 and 0.47 ± 0.29 ml/min, respectively, compared to the methods of stimulated saliva i.e. Salivette® swabs with flow rates of 1.10 ± 0.57 and 1.22 ± 0.46 ml/min for cotton and synthetic respectively, whereas, Greiner Bio-One saliva collection system had the highest salivary flow rates of 2.56 ± 0.95 ml/min.

Holm-Hansen *et al.* (2004), on the other hand, also investigated 8 different commercially available oral fluid collectors: 1) OraSure HIV-1 oral fluid specimen device, 2) UpLink (OraSure), 3) Salivette, 4) Toothette-Plus swabs, 5) BBL CultureSwab orange cap, 6) BBL CultureSwab white cap, 7) BBL CultureSwab red cap (“EZ”) and 8) TRANSORB wicks. The collectors were immersed in water and whole saliva and the collected fluid was removed for investigation. They found no difference in the ability of the collectors to absorb and deliver the fluids, however, the volume of fluid collected varied for each collector. The order of collectors with respect to volume from most to least was: Salivette; Toothette; Orasure; Uplink; Transorb and BBL white. The Orasure oral specimen collection device has also been shown to successfully collect oral fluids in another study investigating the assay of Delta9-tetrahydrocannabinol (THC) (Kauert *et al.,* 2006).

Although many studies have evaluated the collection of oral fluids for protein analysis or other uses (Topkas *et al.* (2012); Holm-Hansen *et al.* (2004); Kauert *et al., (*2006)), the successful collection and subsequent retrieval of oral fluids would also enable the methods

used to provide basis for further investigation for saliva collection with respect to fluoride analysis.

Like the swabs, the use of absorbent discs was demonstrated to collect saliva from soft tissue sites in the mouth, in order to measure fluoride retention (Zero *et al.* 1992). The absorbent discs were placed in different soft-tissue areas of the mouth and samples were collected over a one-hour period. Highest retention of fluoride was found by the tongue and lower posterior vestibule, followed by the upper posterior buccal vestibule and upper anterior labial vestibule. The lowest retention of fluoride was found in the lower anterior vestibule and the floor of the mouth.

Zero *et al.* (1992) also collected unstimulated whole saliva samples following the application of a placebo dentifrice (0.4 ppm), fluoride dentifrice (1100 ppm), fluoride rinse (226 ppm), or fluoride gel (5000 ppm). Whole saliva samples (unstimulated) were collected by asking participants to pool the whole saliva for two minutes. Samples were collected over a 24-hour period. A strong correlation was found between fluoride concentrations in whole saliva and at specific soft-tissue sites. Zero *et al.* (1992) established the oral soft tissues as the major site of fluoride retention in the mouth.

Unstimulated whole saliva is most commonly collected via passive drooling or the spit method (Henson and Wong, 2010). Golatowski *et al.* (2013) also collected unstimulated whole saliva via passive drooling in a 50-ml falcon tube and collected stimulated saliva by 1) asking participants to chew on paraffin gum for 1 minute followed by collecting saliva in a 15 ml falcon tube and 2) using Salivette® cotton swabs, the saliva was obtained by centrifugation at 6000 g for 20 minutes at 4 °C) (n= 9). The highest saliva volume was collected by paraffin gum of 4.1 ± 1.5 ml followed by Salivette® collection, 1.8 ± 0.4 ml and passive drooling, 1.0 ± 0.4 ml. Again, these samples were obtained for proteome analysis, however, they may also be used for fluoride analysis.

Naumova *et al.* (2012) collected whole saliva samples with respect to measuring fluoride concentrations before and after tooth brushing with administration of NaF (1450 ppm) or amine fluoride (1400 ppm). They collected saliva samples by asking participants to spit into plastic tubes for 3 minutes and found the baseline fluoride content of saliva to range from 0.02 ppm to 1.93 ppm. Salivary fluoride concentrations for NaF and amine fluoride ranged from 100.0 to 264.0 ppm and 70.0 to 183.0 ppm, respectively, and returned to baseline levels after 6 hours.

#### 2.3.2.2. Parotid ductal saliva

The Lashley cup has been a common choice for the collection of parotid saliva. Hector and Linden (1987) were able to collect stimulated parotid saliva using Lashley cups adapted from the original method by Lashley (1916). They used Lashley cups which were manufactured using Teflon rod (diameter 16mm, depth 4mm – slightly smaller than Lashley cups by Lashley (1916)) with the inner and outer chambers connected to the flexible collection tube using 30mm of 21 gauze stainless-steel tubing. Parotid saliva was collected with the central chamber placed over the duct with suction applied to the outer chamber using a syringe. The Lashley cup originally manufactured by Lashley (1916) consisted of a metal disc 18 mm in diameter with an inner chamber (10mm diameter, 3mm depth) and outer chamber (2mm width, 3mm depth). The discs were heavily silver plated with silver, soft-drawn tubes attached. Lashley (1916) also used the cup by placing the central chamber over the Stephenson's duct but using a suction pump for suction. More recently, Schroder (2017) used the Lashley cup to successfully collect parotid saliva (n= 40) which indicated that these devices are the most suitable and effective methods for the collection of ductal saliva from the 19$^{th}$ century to date.

#### 2.3.2.3. SL-SM saliva

Nederfors and Dahlof (1993) investigated stimulated SM-SL salivary flow rate using a modified Block-Brottman collection device and compared parotid saliva flow rate using a modified Carlson-Crittenden cups (similar to Lashley cup) (n= 29). They collected both saliva's stimulated using 3% citric acid solution, for 3 consecutive days at 7:30 am and 10:00 am for 2 minutes. Mean stimulated flow rates for parotid and SL-SM saliva were 1.50 +/- 0.83 and 2.25 +/- 1.12 ml/min at 7:30 am and 1.71 +/- 1.16 and 2.54 +/- 1.01 ml/min at 10:00 am, respectively. The found salivary flow rates for SL-SM to be 50% greater than parotid.

The use of a Block-Brottman collection device for SM-SL saliva and Lashley cup (similar to Carlson-Crittenden cup) for parotid saliva was also used successfully by Denny *et al.* (2008). They collected saliva from 33 individuals across 3 different locations.1) University of Rochester: 10 - 100ml saliva was collected for 30 - 120 minutes, stimulated using 0.4% citric acid (n= 3). 2) University of California (Los Angeles) / South California: 2ml parotid and 0.05 - 0.5 ml of SL-SM saliva was collected over a 10 minute period, stimulated using 2% citric acid (n= 10). 3) University of California (San Francisco): saliva was collected over a 30 minute period, stimulated using 2% citric acid.

### 2.3.3. Urine

Urine is the most widely accepted biomarker from the point of view of sample collection. The collection of urine samples is fairly simple, non-invasive, takes minimal time, cost and equipment. Consent is readily obtained, and participants can collect the samples in the comfort of their own private space without the need of any assistance from trained personnel. Urine samples can be collected and stored in sterile plastic bottles in a refrigerator to be analysed within 48 hours or alternatively can be stored in a freezer to be preserved and analysed at a later date.

The most widely used method for urine collection for studies measuring urinary fluoride excretion is '24-hour urine collection', as shown by table 2.7 and discussed in section 2.2.4. Single spot samples have also been used. Agali and Shintre (2016) stated that urinary fluoride concentrations from single spot samples are the most accessible indicators of fluoride exposure. The limitation of spot urine samples for studies on fluoride pharmacokinetics arise from the influence of several factors on the fluoride concentration of a single spot sample. Such factors include normal biological variations among individuals, differences in tasks performed and differences in associated exposure and timing of sample collection in relation to these fluctuations (Bingham and Cohrssen, 2012).

Villa *et al.* (2010) described how the minimum recommended period of time required to estimate daily fluoride excretion should be urinary collection of 24-hours. This would allow to gain a better understanding of the amount of fluoride excreted throughout the day, at various time points along with the effects of any external factors on urinary excretion.

Urine samples collected over 24-hours can also be used to calculate urine flow rate at different times of the day and gain a better understanding of fluoride pharmacokinetics throughout the day. A study by Ekstrand *et al.* (1980) performed urine collection for more than 24-hours. They collected pooled urine 24-hours before and 48-hours after the application of fluoride varnish (5% NaF) in four children, aged 4, 5, 12 and 14 years. The amount of varnish applied was determined by the child's body weight. They found urinary fluoride excretion to be twice as much in older children compared to younger children and this was in accordance to the dose provided. Daytime urinary fluoride excretion was found to be 100 µgF/12-hours and 200 µgF/12-hours prior varnish application in young and old children, respectively. Following varnish application, urinary fluoride levels were found to be 550 µgF/12-hours and 1100 µgF/12-hours in young and old children, respectively. Ekstrand

*et al.* (1980) stated that urinary output data can provide an insight into the amount of fluoride absorbed into the blood stream or the bioavailability of the source of fluoride administered, which in this case was the fluoride varnish.

Another study performing urine collection for more than 24-hours was reported by Ketley and Lennon (2000) who collected individual urine samples for a continuous period of 55 hours from 4 to 5-year-old school children (n= 8) children participating in a school milk fluoridation scheme, as discussed in section 2.2.4. The mean daily urinary fluoride excretion was found to be 0.33 mg/day, under usual conditions of fluoride intake (i.e. milk containing 0.5 mg fluoride, customary diet and toothbrushing with fluoride toothpaste).

The time period of 24-hours is said to be reliable for urine collection, independent of dietary habits, timing of meals and maximal fluoride intake (Marthaler, 1999). However, Marthaler (1999) does also acknowledge that collection of 24-hour urine may not be practical or feasible to collect in certain circumstances, especially when involving young children. Zipkin *et al.* (1956) stated how there are challenges associated with the collection of 24-hour urine. Although, thought that concentrations in single spot samples may vary from 24-hour samples, they found no statistical difference between the spot samples collected from 9 adult males and the 24-hour urine sample. Moreover, the fluoride/creatinine ratio of spot samples can be used to estimate mean 24-hour urinary fluoride excretion (Zohouri *et al.,* 2006). Zohouri *et al.* (2006) collected both 24-hour and single morning spot urine samples from young children aged 16 - 36 months (n= 7) and found a positive correlation (Pearson's correlation = 0.76) between estimated fluoride excretion (using the fluoride/creatinine ratio of spot urine) and measured fluoride excretion in the 24-hour urine sample.

## 2.4. Fluoride analytical methods

The methods used to analyse fluoride from samples involve several stages. Firstly, the samples are required to be pre-treated. Preparation is vital to release the bound forms of fluoride, convert inorganic fluoride into organic fluoride, remove other ions / compounds or material that may interfere with electrode analysis. Techniques used to prepare samples for fluoride analysis including open ashing, fusion, and confined combustion procedures such as oxygen flask, oxygen bomb, tubular furnace or oxyhydrogen flame (Venkateswarlu 1990; Venkateswarlu 1994). TISAB buffer is required to adjust the pH level, avoiding hydroxide interference and displacing any bound fluoride (Frant and Ross, 1968).

Malde *et al.* (2001) used sodium hydroxide (NaOH) as an ashing aid to prepare food samples for the determination of fluoride. They recommended the use of 2.5 ml or 5 ml 8 mol/l NaOH, depending on sample weight. The samples were dried on a hot plate before ashing in a muffle furnace at 200°C for 16 hours, followed by another 3 hours at 525 °C. Ashed samples were neutralised using hydrochloric acid and then required to be dissolved in distilled water as well as using TISAB III buffer to adjust the pH to 5.2 - 5.4 before analysing for fluoride. The method of ashing and un-ashing was also used to determine fluoride in food samples by Taves (1983) who found a difference greater than 3 nmol/g of fluoride in ashed samples compared to un-ashed for two types of cereal and black pepper samples. Ashing as a procedure does however have its limitations. It can lead to the loss of fluoride as well as being prone to fluoride contamination. These limitations can be overcome in biological materials by the use of the oxygen bomb technique where fluoride in the bomb washings is determined with the use of the F-ISE, along with reverse extraction of fluoride, if required (Venkateswarlu, 1975)[B].

Another example of sample preparation of fluoride biomarkers for analysis was demonstrated by Gron *et al.* (1968) who found that better results of analysing fluoride in parotid saliva were obtained when the pH of the saliva sample was adjusted to 4.7 - 4.8 before measuring. A study mentioned previously by Parkins *et al.* (1974) used a buffer i.e. 1mM trisodium citrate (ratio 1:1) to adjust the pH and ionic strength of the plasma sample gained from adult patients aged 17 - 80 years (n= 41) in a hospital setting to compare with fluoride concentrations in bone. The plasma sample was then analysed using the fluoride ion-selective electrode.

Following pre-treatment, samples require to be separated from interfering substances. Such methodologies have been outlined by Venkateswarlu (1990) and Venkateswarlu (1994) as most preferred for determining fluoride from the following biological materials, 1) Biological fluids i.e. blood, urine, saliva, etc.: fluoride electrode (with suitable buffer); hanging-drop fluoride electrode; diffusion; calcium phosphate adsorption; reverse extraction and spectrophotometry, 2) Dental plaque fluid: micro-fluoride electrode, 3) Soft tissue: diffusion; fluoride electrode; reverse extraction and hanging-drop fluoride electrode, 4) Calcified Tissue: dissolved ashing with fluoride electrode; ashing with diffusion and fluoride electrode / spectrometry; ashing with reverse extraction / spectrometry; and no ashing with reverse extraction and fluoride electrode.

It is essential that a suitable low fluoride blank sample is obtained during fluoride analysis. The fluoride blank largely influences the validity of the fluoride analysis results (Glick, 2009). The final stage involved in fluoride analysis is measurement of the fluoride concentration after pre-treatment and separation of the sample. Several methods have been used for the determination of fluoride. The type of method used is dependent on the sample type. Samples whether biological or non-biological are often analysed using potentiometric (Fluoride Ion Selective Electrode (F-ISE)) and gas chromatographic methods (Clement International Corporation, 2001 and Malde *et al.*, 2001). Other methods for fluoride analysis also include calibration graphs, standard additions procedures (Villa, 1979), volumetric, colorimetric, ion chromatography and non-destructive nuclear methods (Lindahl, 1983). The fluoride ion specific electrode is said to be the most common method for fluoride analysis (Lindahl, 1983).

Martinez-Mier *et al.* (2011) developed gold standard F-ISE methods for fluoride analysis which included the Direct Analysis method using F-ISE / pH ion meter and Hexamethyldisiloxane (HMDS) micro-diffusion method. Table 2.8 outlines the samples suitable for the two gold standard methods, as recommended by Martinez-Mier *et al.* (2011). Greater amounts of fluoride have been found to be recovered when using the diffusion method i.e. 96 - 100% compared to the direct method i.e. 67 - 90% when analysing fluoridated salt (Martinez-Mier *et al.,* 2009).

**Table 2.8. Recommended samples for the Direct Analysis method using F-ISE / pH ion meter and Hexamethyldisiloxane (HMDS) micro-diffusion method. (adapted from Martinez-Mier *et al.* (2011)).**

| Method | Direct Analysis method | HMDS micro-diffusion method |
|---|---|---|
| **Sample** | Saliva | Plasma |
| | Urine | Saliva |
| | Beverages | Urine |

| | | |
|---|---|---|
| | | Food |

Biological samples such as blood plasma and ductal saliva tend to be lower in volume compared to urine and require reliable micro-methods for the determination of fluoride. Ekstrand (1977)[A] developed a micro-method for the determination of fluoride in plasma and saliva. The method was based on the known addition-slop determination technique using F-ISE. Ekstrand (1977)[A] found it was possible to analyse samples as small as 150 µl in volume and as low as 4 ng/ml fluoride in concentration, using good reproducibility. Venkateswarlu (1975)[A] on the other hand, developed a micro-method using a hanging drop fluoride electrode for the determination of bodily fluids from samples as small as 5 µl using a sodium acetate buffer (pH 4.8). Vogel *et al.* (1990) demonstrated a variety of techniques suitable for analysing fluoride from nano-liter and micro-liter sized liquids ranging 0.005 µl to 5 µl. The methods used involved: 1) micropipette procedures for transference and dilution of samples; 2) construction of miniature and micro fluoride-selective electrodes and 3) methods for adapting standard electrodes for micro- and semi-micro volumes.

More recently, the method of gas chromatography-mass spectrometry and solid-phase micro-extraction has also been developed for biological samples (Kwon and Shin, 2015). Kwon and Shin (2015) were able to detect fluoride as low as 9 and 11 µgF/L in 1.0 ml of plasma and urine, respectively. Although, they deemed this method to be simple, the gold standard methods by Martinez-Mier *et al.* (2011) are most preferred.

With advances in technology and instrumentation, an online membrane-based distillation coupled with ion chromatography method was used successfully by Lou *et al.* (2017) for the automatic detection of trace fluoride in serum and urine samples. They used the membrane-based distillation (Hydrophobic polytetrafluoroethylene (PTFE) hollow fiber membrane) directly in serum and urine samples in order to eliminate matrix interferences and enrich fluoride followed by using ion chromatography to determine fluoride levels. Lou *et al.* (2017) also proposed that the use of this method may have wider applications for fluoride detection from complex samples which is a modern advance in the field of fluoride analysis.

Many of the current methods for fluoride analysis are straightforward and do not involve extensive equipment, time or cost. Examples of such include the gold standard direct fluoride ion selective electrode method and the Hexamethyldisiloxane (HMDS) micro-diffusion method. Some of the great advantages of using the fluoride ion selective electrode include its insensitivity to colour (Bratovcic *et al.,* 2009) which is a great benefit for samples

which are suitable for the direct method and hence don't require the sample to be pre-treated for analysis. Other advantages include lack of interference and reactivity with the sample, electrode efficiency and selectivity (Bratovcic and Odobasic, 2011).

Although the methods used to analyse samples for fluoride have many advantages, there are also downfalls. Many samples require preparation to be suitable for analysis using the methods described above especially with the most preferably used ion selective electrode. This can often lead to loss of sample size and sample contamination which is of great risk when samples of small volume are involved (Clement International Corporation, 2001). The equipment involved is often sensitive and fragile and requires calibration. Even more so, the samples involved require standardisation to regulate ion-activity and a buffer solution such as TISAB reagent is used to fix ion strength (Bratovcic *et al.,* 2009). However, the advantages outweigh the disadvantages and methods such as the Direct Analysis method using F-ISE / pH ion meter and Hexamethyldisiloxane (HMDS) micro-diffusion method withhold their reputation as the 'gold standard' methods.

The validity and reliability of the fluoride concentration of a sample and hence the method used for analysis can be tested by 1) re-analysis and 2) recovery, of 10% of the samples (Zohoori and Maguire, 2015). Fluoride recovery involves the addition of a known concentration of fluoride standard to the sample for analysis. If the added fluoride is recovered along with the samples original fluoride content, the method / technique used for fluoride determination is deemed to be accurate and the fluoride content of the sample must be correct. Retief *et al.* (1985) used the methods of F-ISE and gas chromatography for fluoride recovery to demonstrate the accuracy of their methods. The use of F-ISE based standardized protocols have been shown to increase fluoride recovery from 78% to 89.05%, as demonstrated by Martinez-Mier *et al.* (2011).

## 2.5. Physical Activity and Exercise

The World Health Organisation (2014) defined physical activity as 'any bodily movement produced by skeletal muscles that requires energy expenditure'. Exercise, on the other hand, is defined as a 'subcategory of physical activity that is planned, structured, repetitive, and purposeful with the intention of improving or maintaining one or more components of physical activity'. In the wider context, physical activity is termed as a physiological state,

whereas exercise is often described as a set of behaviours and their context / purpose (Ayers *et al.,* 2007).

There are many health benefits of regular moderate to vigorous physical activity including reduction on cardiovascular disease, hypertension, diabetes mellitus (type 2), obesity, thromboembolic stroke, colon cancer, breast cancer, anxiety and depression (Bouchard, 2001).

Improvement in cardiometabolic makers are not usually seen in children as physical activity effect of these markers are only seen later in life (Biddle, 2004). There is however, strong evidence to conclude the beneficial effects on adiposity, musculoskeletal health and fitness and cardiovascular health in school children and youth (Janssen and LeBlanc, 2010).

The recommended amount of physical activity for healthy adults (aged 18 to 65 years) to promote and maintain health is: A) 30 minutes of moderate-intensity aerobic physical activity for a minimum of five days per week; or B) 20 minutes of vigorous-intensity aerobic physical activity for a minimum of three days per week (Haskel *et al.,* 2007). On the other hand, the recommendation for children aged 2 and above, as stated by the American Heart Association (AHA) (2014) is a minimum of 60 minutes of moderate to vigorous aerobic exercise.

Janssen and LeBlanc (2010) conducted an extensive systematic review and were able to associate dose-response relations in physical activity and health. They also recommended an average of at least 60 minutes a day and several hours of moderate activity as well as more vigorous activities on at least 3 days of the week for children aged 5 - 17 years in order to achieve health benefits, although some benefits may be achieved through an average of 30 minutes of moderate to vigorous physical activity a day.

This is supported by Strong *et al.* (2005) and World Health Organisation (2011) which in addition stated that these recommendations can be met in shorter bouts each day, as well as highlighting that muscle and bone strengthening, moderate to vigorous activities should be incorporated for 60 minutes or more daily.

### 2.5.1. Acute and chronic effects of exercise

Exercise has both acute and chronic (or training) effects on health and wellbeing. Acute effects refer to any immediate physiological response following exercise, and chronic effects

result from long term consistent exercise. The ranges of acute physiological effects are well known on blood lipids, blood pressure, glucose homeostasis, insulin sensitivity, hemostasis, vascular reactivity and immunological function (Thompson *et al.*, 2001 and Kesaniemi *et al.*, 2001). The chronic effects of regular exercise benefit the muscles, circulo-respiratory system, skeletal system, energy systems, etc. Table 2.9. summarises some of the acute and chronic effects of exercise (Baechle and Earle, 2008).

**Table 2.9. Summary of acute and chronic physiological responses of exercise. (Adapted from Baechle and Earle (2008)).**

| Type of response | Acute or Chronic | Physiological response |
|---|---|---|
| Cardiovascular | Acute | • Increased cardiac output<br>• Increased stroke volume |
| | Chronic | • Increased maximal cardiac output<br>• Decreased heart rate |
| Respiratory | Acute | • Increased ventilation<br>• Increased breathing frequency |
| | Chronic | • Increased tidal volume and breathing frequency |
| Gas | Acute | • Diffusion capacity of oxygen and carbon dioxide increased |
| Muscular | Chronic | • Increased aerobic capacity of trained musculature<br>• Increase in size and number of mitochondria and myoglobin |
| Bone and Connective tissue | Chronic | • Improved bone mass<br>• Tendon, ligament and cartilage strength |
| Endocrine | Chronic | • Increase in hormonal circulation |

In general, the acute effects of exercise last no more than three days and to maintain acute benefits; exercise must be repeated at least every three days (Lemura and Duvillard, 2004). Blood pressure can decrease approximately 5 - 7 mm Hg following exercise and the mechanisms thought to induce this change include neurohumoral, vascular, and structural adaptations; decreases in catecholamines and total peripheral resistance; improved insulin sensitivity; and alterations in vasodilators and vasoconstrictors (Pescatello *et al.,* 2004). With respect to blood pressure, the acute effects post exercise can last up to 12-16 hours (Thompson *et al.,* 2001) and heart rate generally remains elevated post exercise.

Cardiac output can increase up to four times the resting value from 5 litres/minute to 20 - 22 litres/minute, during maximal exercise (Baechle and Earle, 2008). The acute response of exercise on the skeletal muscles arises from the increased requirement for nutrients and oxygen which increases blood flow to the muscles. The chronic response, on the other hand, is improved cardiovascular function and increased cardiac output which in turn increases the blood flow capacity in skeletal and cardiac muscle (Whyte and Laughlin, 2010).

Changes to concentrations of triglycerides, total cholesterol or high density lipo-protein cholesterol (HDL-C) in plasma may also be seen immediately after exercise (Bouchard *et al.,* 2012). Endurance exercise has been positively associated with increases in HDL-C levels in men and these effects may differ for each individual dependant on the intensity, duration and frequency of exercise; initial HDL-C levels and length of training (Vella *et al.,* 2001).

In relation to bone mass, mineral density and remodelling, chronic effects of exercise are associated with higher bone mass in children (Maimoun and Sultan, 2011) and adolescents (Gracia-Marco *et al.,* 2011) whilst decreasing bone loss in elderly (Shedd *et al.,* 2007). The acute effects on the other hand, have no immediate measurable effects with respect to bone turnover (Maimoun and Sultan, 2011).

The effect of resistance exercise has shown to exert significant acute hormonal responses. Hormones including growth hormones (GH) and anabolic hormones e.g. testosterone are known to increase during the first 15 - 30 minutes post-resistance exercise. Such acute responses are deemed more critical to tissue growth and remodelling compared to chronic changes in hormone concentrations (Kraemer and Ratamess, 2005).

The acute metabolic effects of exercise for any individual can be affected by many factors including but not limited to, baseline cardiorespiratory fitness; pre-exercise lipid levels; duration and intensity of exercise; and change in dietary composition. Whereas, the chronic effects of exercise may be affected by the same factors as acute exercise, plus volume of training; exercise mode (e.g. aerobic vs resistance training); or changes in body weight (McKeag and Moeller, 2007).

### 2.5.2. Response of exercise at:

- **Different Intensity**

Exercise intensity reflects the energy requirements for an activity per unit time and specific energy systems activated. It is most commonly defined using metabolic equivalent (MET) which is the resting metabolic rate, so the amount of oxygen consumed whilst sitting at rest and is equal to 3.5 ml $O_2$ per kg bodyweight x minutes.

Light (<4 METs), moderate (4 - 6 METs) and vigorous intensities (>6 METs) all have different effects on the physiological processes within the human body (Jette *et al.*, 1990; Kraemer *et al.*, 2012). Relating the endocrine system, many of the processes are only stimulated at higher intensities of exercise, such as the secretion of growth hormone (Juul and Jorgensen, 2000), blood cortisol levels and aldosterone levels (Kraemer *et al.*, 2011).

With respect to the respiratory system, an increase in ventilation during low work intensities results primarily from an increase in tidal volume. The respiratory rate is also increased during high work intensities. Ventilation rates can reach up to 200 litres/minute at maximal rates of work, compared to 10 litres/minute at rest (Manley, 1996). However, breathing capacity does not reach its maximum even during vigorous exercise (Burton *et al.*, 2004).

An increase in accumulation of lactic acid and subsequently partial pressure $CO_2$ following vigorous exercise is caused by insufficient oxygen which increases the reliance on glycolysis. The stimulation of ventilation counteracts this, consequently leading to a decrease in partial pressure $CO_2$ which provides respiratory compensation for further lactic acid production, preventing a decline in blood pH. This homeostasis remains almost constant during light and moderate exercise (Burton *et al.,* 2004).

A study by Van Loon *et al.* (2001) investigated the regulation of muscle fuel selection in cyclists (n= 8) during rest and during three consecutive 30-minute stages of exercise at intensities of 40%, 55% and 75% maximal workload ($W_{max}$). They found an increase in

muscle glycogen and plasma glucose oxidation rates with every increment in exercise intensity. Whole-body fat oxidation increased at 55% $W_{max}$ but declined at 75% $W_{max}$. They attributed the reduction in fat oxidation during high-intensity exercise to a downregulation of carnitine palmitoyl-transferase I, either by a marked decline in free carnitine availability or by a decrease in intracellular pH.

A study by Tremblay *et al.* (1994) showed high-intensity intermittent-training to induce a greater reduction in subcutaneous adiposity compared to endurance training with moderate intensity. This may be explained by the high demand imposed on the glycolytic energy metabolism making subjects experience high muscle lactate concentrations after repeated and maximal exercises of short duration (Irving *et al.,* 2008; Yoshioka *et al.,* 2001).

Children and adolescents in contrast to this, show smaller reductions in intramuscular pH during high intensity exercise (Boisseau and Delamarche, 2000). Such smaller reductions in pH may produce smaller effects of the related consequences such as fluoride metabolism in children compared to adults. A study by Boisseau and Delamarche (2000) also explained the lower glycolytic capacity and limited production of muscle lactate in prepubertal children could be caused by reduced activity of phosphofructokinase-1 and lactate dehydrogenase enzymes which may be related to a reduced sympathetic response to exhaustive resistance exercise in young people. This has an overall impact resulting in a longer duration of physical activity that can be maintained by younger children in comparison to adults. Overall, the majority of physiological responses to exercise are intensity dependant.

- **Different Volume**

The volume of exercise relates to the frequency, intensity and duration of exercise. It can be measured by the number of times each activity is performed multiplied by the energy expenditure and is often expressed at MET-hours (Kokkinos, 2009). There is said to be a dose-response association between the volumes of exercise and health / fitness outcome i.e. higher volumes of exercise leading to greater health and fitness levels (American College of Sports Medicine, 2013).

The role of exercise volume in general is often underestimated in comparison to exercise intensity. Although higher intensities of exercise are important to optimally stimulate muscle protein synthesis, a study by Burd *et al.* (2010) suggested that the extent of muscle protein synthesis is not entirely dependent on load but is also related to exercise volume. They

found that low-load high volume exercise was more effective in inducing acute muscle anabolism than high-load low volume. Large volumes of exercise are also associated with greater fat loss (Tremblay *et al.,* 1994). Tremblay *et al.* (1994) investigated two different modes of exercise training on body fatness and skeletal muscle metabolism in young adults: 1) 20-week endurance training (n= 8 male and 9 female) or 2) 15-week high-intensity intermittent training (n= 5 male and 5 female). They found vigorous exercise to favour negative energy and lipid balance more than low-moderate exercise. Also, the metabolic adaptations of skeletal muscle in response to high-intensity intermittent training were found to favour the process of lipid oxidation.

- **Acute and Chronic sessions**

Acute and chronic sessions of exercise have an effect on overall health with the physiological responses often having more promising health benefits in the long term. An example is shown by a study from Cardoso *et al.* (2010) that demonstrated acute exercise was found to lower ambulatory blood pressure in hypertensive subjects; whereas, chronic exercise was found to be an effective strategy to lower blood pressure in both normotensive and hypertensive subjects.

With respect to cardiac output, blood flow to skeletal muscle and skin during acute sessions of exercise is gained by approximately 80% cardiac output, compared to 20% during rest. Active skeletal muscles receive more blood during exercise not only from the increased cardiac output but also by the redistribution of blood flow away from areas of low demand, whereas, the skin receives more blood flow due to an increase in body temperature (Manley, 1996). Chronic sessions of exercise, on the other hand, induce greater cardiac output distribution to the skin over time in order to counter the increasing body temperature, especially in hot and humid temperatures. This, in turn, limits the amount of blood flow distributed to skeletal muscle and exercise endurance (Manley, 1996).

During acute sessions of exercise, pulmonary ventilation increases immediately primarily through stimulation of the respiratory centres in the brain and following feedback from the proprioceptors in the muscles and joints of the active limbs. During chronic sessions of exercise, pulmonary ventilation is increased further stimulated by increases in $CO_2$ production and hydrogen ions (H+) along with blood and body temperature (Manley, 1996).

Following acute (moderate) exercise, an increase in serum immunoglobulin levels are witnessed resulting from extravascular protein pools and an increased lymph flow, although plasma volume does not change. Chronic exercise, oppositely, has been associated with decreased serum immunoglobulin levels compared to acute exercise. The link between chronic exercise and muscle cell damage and local inflammation has well been established. Therefore, the reduced immunoglobulin levels may result from the use of autoantibodies which assist macrophages in the disposal of muscle cell breakdown followed by their clearance from the blood (Nieman and Nehlsen-Cannarella, 1991).

A study by Cipryan *et al.* (2017) compared the acute and post-exercise differences in cardiorespiratory, metabolic, cardiac autonomic, inflammatory and muscle damage responses to high-intensity interval exercise (HIIT) between endurance (n= 8) and sprint athletes (n= 8). However, they did not find any considerable differences in markers of cardiac autonomic regulation, inflammation and muscle damage between endurance (chronic exercise) and sprint athletes (acute exercise).

Acute and chronic sessions of exercise also have an impact on the endocrine system e.g. Growth hormone (GH) is stimulated by acute exercise. Chronic exercise at higher intensities which leads to greater fitness levels may further enhance GH secretion (Juul & Jorgensen, 2000). Overall both acute and chronic sessions of exercise have a positive effect on health.

## 2.6. Association between Physical Activity and Fluoride Metabolism

There is limited scientific peer-reviewed literature around the effects of exercise on fluoride metabolism. Based on the grey literature i.e. information produced by organisations outside of the traditional publishing and distribution channels, exercise has been suggested as a fluoride detox (Dr Axe, 2018). Although regular exercise has been recommended to prevent gum disease (Carefree Dental, 2016), a possible higher risk of tooth decay has also been linked to exercise due its effect on increasing suggested the alkalinity of saliva and a significant decrease in salivary flow rate which in turn will host a greater environment for the growth of harmful bacteria (Dentistry Today, 2014). Several sources have linked exercise with poor oral health, especially for athletes (Medical Daily, 2014; Reynolds, 2014; Carefree Dental, 2016; Piedmont Smiles, 2017).

The mechanisms by which exercise could affect fluoride metabolism are still unknown. Depending on the balance of several factors, exercise could be associated with either

decreased or increased circulating fluoride levels (Buzalaf, 2011). One of the possible mechanisms is that the decrease in blood flow and the increase in lactic acid concentration in the blood during moderate and vigorous exercise can reduce glomerular filtration and urinary pH (Freund, 1991). These physiological adaptations may reduce fluoride excretion and increase renal reabsorption. A study performed on rats, demonstrated reduced fluoride toxicity levels after physical activity combined with thermo-neutral temperatures (Basha and Sujitha, 2012). Basha and Sujitha (2012) gave three-month-old male rats drinking water containing 600 ppm NaF for one month. The rats performed swimming exercises at temperatures of 20°C, 25°C, 30°C, and 35°C and were found to show fluoride induced oxidative stress in tissues of the brain, heart, liver and kidney.

Another study showed lower plasma fluoride levels in rats following exercise which was probably induced by an increase in fluoride uptake by bone (Lombarte *et al.,* 2013). Thirty rats were divided into 3 groups (n= 10 per group): 1) control i.e. drinking water with no NaF, 2) drinking water with 15mg/l NaF for 30 days and 3) drinking water with 15 mg/l NaF for thirty days and daily exercise on a treadmill for 60 minutes at a speed of 2.25 m/min. An increase in insulin resistance was seen in rats treated with 15 mgF/l drinking water (but no exercise), whilst a decrease was seen in insulin resistance along with increase in bone in rats receiving the 15 mgF/l drinking water with exercise.

In contrast to this, decreased fluoride absorption in inactive tissues such as bones, gastrointestinal tract, etc. may result from a reduction in blood flow in such tissues during exercise. It is also well known that catecholamines released during exercise induce renal vasoconstriction and reduce kidney blood flow. Moreover, the response of catecholamines is dependent on the intensity and duration of exercise (Garrett and Kirkendall, 2000).

However, at the same time, prolonged sessions of physical activity stimulate an increase in lactate concentration which leads to a reduction in pH gradient across cell membranes which promote the diffusion of fluoride (as HF) from extracellular to intracellular fluid (Buzalaf, 2011), thus increasing fluoride absorption in most tissues. Although fluoride tends to move from a low to high pH, this pathway may reverse in the muscles.

A study by Frese *et al.* (2015) who found an increased risk of dental erosion in athletes (n= 35) compared to non-exercising individuals (n= 35). A significant correlation was found between caries prevalence and the cumulative weekly training time in athletes ($r = 0.347$, $p = 0.04$). They also performed saliva assessments on athletes undertaking an incremental

running field test (IRFT) (n= 15) and found and significant increase in salivary pH after the IRFT.

# Chapter 3. Aims and Objectives

## 3.1. Overall Main Aim:

The overall main aim of this thesis was to investigate the effects of exercise on fluoride metabolism in human subjects.

**Overall objectives**:

- To assess practical and acceptable methods for the collection of ductal saliva for studies on fluoride metabolism in humans (Study 1).
- To assess practical methods of blood and saliva collection for studies on fluoride metabolism in humans (Study 2).
- To investigate fluoride absorption and excretion by measuring levels of fluoride in plasma and urine before and after no, light, moderate and vigorous continuous exercise in adults (Study 3).
- To develop a protocol for assessment of the effects of an acute session of high-intensity free-living activity on fluoride excretion in children (Study 4).

## 3.2. Specific Aims and Objectives:

### 3.2.1. Study 1: Practicality and suitability of Dry Tips and Lashley cups for the collection of saliva: a preliminary investigation.

The main aim of this preliminary investigation was to assess the use of Dry Tips and Lashley cups for the collection of ductal saliva for studies on fluoride metabolism in humans.

**Objectives:**

- To determine the endogenous fluoride content of Dry Tips using ashing and/or liquid nitrogen crushing techniques.
- To investigate the recovery of added fluoride from the Dry Tips using ashing and/or liquid nitrogen crushing techniques.
- To investigate the use of Dry Tips in collecting ductal saliva.

- To investigate the use of Lashley cups in collecting ductal saliva.
- To compare the efficiency of measuring fluoride from ductal saliva obtained using Dry Tips compared with Lashley cups.

### 3.2.2. Study 2: Determination of fluoride in saliva and plasma: sample collection and analysis.

The aims of this study were:

- To collect blood samples and investigate the association between fluoride concentrations in whole blood and plasma (Study 2A).
- To collect blood and saliva samples and investigate the relation between fluoride concentrations in blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva (Study 2B).

**Objectives**

**Study 2A: Association between fluoride concentrations in whole blood and plasma.**

- To investigate the practicality of venous blood sampling by venepuncture.
- To investigate the practicality of capillary blood sampling by dermal puncture of fingertip (finger prick).
- To measure fluoride concentrations of whole blood samples collected by 1) venous and 2) capillary (finger prick) blood sampling.
- To compare fluoride concentrations of venous and capillary whole blood.
- To measure fluoride concentrations of plasma samples collected using venous blood sampling.
- To compare fluoride concentrations of whole blood and plasma collected using venous blood sampling.

**Study 2B: Relation between fluoride concentrations in blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva.**

- To investigate the use of oral syringes in the collection of SL-SM saliva.
- To measure the fluoride concentration of blood plasma samples.
- To measure fluoride concentrations of SL-SM saliva.

- To measure fluoride concentrations of whole saliva.
- To compare fluoride concentrations of SL-SM saliva and whole saliva.
- To compare fluoride concentrations in blood plasma and saliva.

### 3.2.3. Study 3: The effects of different intensities of continuous exercise on fluoride metabolism in adult humans.

The main aim of this study was to investigate the effect of different intensities of continuous exercise on fluoride metabolic responses in adults.

**Objectives**:

- To investigate fluoride absorption by measuring levels of plasma fluoride before and after different exercise intensities.
- To measure urinary fluoride excretion (UFE) rate before and after different exercise intensities over 24-hour period.
- To investigate the relationship between fluoride absorption and excretion at different exercise intensities.

### 3.2.4. Study 4: Development of a practical and ethically approved protocol to investigate the effects of exercise on fluoride excretion in children

The overall aim was to develop a feasible and ethically sound protocol for an experimental study on the effects of exercise on fluoride excretion in children.

**Objectives**:

- To develop a protocol framework to assess the effects of exercise on fluoride metabolism in children.
- To develop a protocol that is ethically sound.
- To pilot the child project in order to assess the feasibility of the protocol.

# Chapter 4. Study 1: Practicality and suitability of Dry Tips and Lashley cups for the collection of saliva: a preliminary investigation.

## 4.1. Introduction

Assessment of fluoride body burden involves evaluation of fluoride in the body tissues (e.g. bone, teeth) and fluids (e.g. urine, plasma and saliva). Bone contains 99% of total fluoride in the body and plasma has been suggested as an indicator of bone fluoride levels (Carvalho *et al.,* 2006). From a pharmacokinetics perspective, plasma is referred to as the "central component in which fluoride passes for distribution and excretion. Pharmacokinetic studies of fluoride may involve repeated venous blood sampling, which is the most invasive procedure within pharmacokinetic studies. The dangers of repeated blood sampling include pain, an increased risk of infection, excessive bleeding, bleeding under the skin (bruising or more severely, hematoma) and risk of fainting (Buowari, 2013).

Due to the reported proportional relation between fluoride concentration in plasma and parotid / submandibular ductal saliva (Oliveby e*t al.*, 1989), ductal saliva, as a less invasive method, could be an alternative to the plasma for fluoride studies. Therefore, it is important to investigate the practicality of the collection of ductal saliva as a substitute alternative for plasma.

The current techniques used to collect ductal saliva from the parotid, sublingual and submandibular ducts often involve the use of collection devices (Fukushima *et al.,* 2011; Oliveby *et al.,* 1989), as discussed in Chapter 2, section 2.3.2. Some of which are not widely used or commercially available. Therefore, due to the necessity of finding easy and inexpensive methods for the collection of ductal saliva, this study aimed to assess and develop the use of Dry Tips (commercially available) and Lashley cups (previously used; Fukushima *et al.*, 2011) for the collection and determination of fluoride in ductal saliva.

## 4.2. Aims and Objectives

The main aim of this preliminary investigation was to assess the use of Dry Tips and Lashley cups for the collection of ductal saliva for fluoride pharmacokinetic studies, which are important to determine the correct dose of fluoride, in children and adults.

**Objectives:**

- To determine the endogenous fluoride content of Dry Tips using ashing and/or liquid nitrogen crushing techniques.
- To investigate the recovery of added fluoride from the Dry Tips using ashing and/or liquid nitrogen crushing techniques.
- To investigate the use of Dry Tips in collecting ductal saliva.
- To investigate the use of Lashley cups in collecting ductal saliva.
- To compare the efficiency of measuring fluoride from ductal saliva obtained using Dry Tips compared with Lashley cups.

## 4.3. Materials and Methods

### 4.3.1. Determination of endogenous fluoride content of Dry Tips

The method involved preparation of Dry Tips followed by fluoride analysis using the hexamethyldisiloxane (HMDS) acid-diffusion method.

#### 4.3.1.1. Preparation of Dry Tips

Three preparation methods were tested:

1) Direct Ashing: The technique of open ashing (Whitford *et al.*, 1999) was used to prepare the Dry Tip samples for fluoride analysis. Dry Tip samples were weighed and placed into pre-weighed crucibles. The crucibles were then placed in a muffle furnace (Vecstar ECF3, Chesterfield) at 600°C for 35 minutes. The crucibles were weighed again after ashing to measure the weight of the ashed sample. This method was ran using both ceramic and nickel crucibles.

2) Using sodium hydroxide (NaOH) as an ashing aid before ashing: 8M NaOH was added to the Dry Tip sample (Ratio 3:1 NaOH to sample) (Malde *et al.,* 2001). This sample was then dried on a hotplate before placing in the muffle furnace at 600°C for 35 minutes.

3) No ashing: Dry Tip were directly set up for fluoride analysis without any prior ashing.

**a)** The Dry Tip samples were set up whole.

**b)** The Dry Tip samples were cut into small pieces using scissors to increase the surface area of the Dry Tip and increase contact with acid during the HMDS acid-diffusion method.

#### 4.3.1.2. Fluoride analysis

The ashed and un-ashed Dry Tip samples were then analysed using the HMDS acid-diffusion method. This method is a validated method previously published in detail by Martinez-Mier *et al.* (2011):

1. Ashed samples: The weight of the ashed samples was measured, and each sample was divided into three subsamples (three replicates) in pre-weighed petri-dishes. This was followed by measuring the final weight of each subsample (i.e. replicate).
2. Un-ashed (Whole): One pre-weighed whole Dry Tip was placed in a petri-dish. This was performed in triplicate for each analysis.
3. Un-ashed (Cut): One pre-weighed Dry Tip was cut into small pieces using scissors and placed in a petri-dish. This was performed in triplicate for each analysis.
4. Standards: A set of standards were set up using 1 ml of 0.01, 0.1, 1, 10 and 100 ppm fluoride in triplicate.

The weight of all samples including standards was made up to 3 ml or 3 grams (g) with the addition of double de-ionised water ($ddH_2O$). A NaOH trap was prepared by pipetting 50 µl 0.05 N NaOH in 4 - 5 drops on the inside of the petri-dish lid. The lid (pre-prepared with a drilled hole) was then sealed onto the petri-dish using parafilm and 1 ml of 3 N HMDS-saturated sulphuric acid was pipetted into the hole followed by immediately sealing the hole. The samples were left at room temperature overnight. The following day, the petri-dishes were unsealed, and the plate contents were discarded. The NaOH drops inside the lid were collected and 25 µl 0.1 N acetic acid was added. This sample was analysed using the

Fluoride Ion Selective Electrode (Model Orion 9609BNWP, Thermo Scientific, USA) and meter (Model Orion 720A+, Thermo Electron Corporation, USA).

### 4.3.2. Recovery of added fluoride

To investigate the recovery of added fluoride from Dry Tips, the pads were pre-weighed and either 1.5 ml of $ddH_2O$ or fluoride standard (0.5 ppm or 1 ppm) was added to the pad. Once the liquid was fully absorbed, the Dry Tip weight was measured again and prepared for fluoride analysis using the methods mentioned in section 4.3.1.1.

### 4.3.3. Collection of Saliva samples using Dry Tips and Lashley Cups

#### 4.3.3.1. Ethical approval

This project was approved by the Research Governance and Ethics Committee, School of Health and Social Care, Teesside University (Reference number: 066/15 - approval attached – Appendix 1). The complete ethics application with supporting documents are also attached (Appendix 2).

#### 4.3.3.2. Collection of saliva samples using Dry Tips

Twenty-five participants were recruited from staff and students at Teesside University and their family and friends. An invitation email with the participant information sheet attached was circulated by the researcher (Maria Sajjad) via the school administrator. Those participants interested in taking part contacted the researcher by telephone or email.

Participants were then invited to attend a meeting immediately prior to data collection where the nature and design of the study was discussed, and participants were given the opportunity to ask any questions or concerns they had regarding the research before obtaining informed consent.

Saliva sampling was performed by an experienced dental nurse from the Dental Hospital, Newcastle University, Newcastle. Before sampling, the mouth was rinsed using 100 ml deionised water and the inner cheek (surrounding buccal mucosa) was cleaned with a gauze moistened with deionized water and then dried. Commercially available Dry Tips (Figure 4.1) were placed over the Stephenson’s duct of the left and right cheek and salivary flow rate was stimulated using a few drops of lemon juice with a swab wiped on the tongue

at 30 second intervals for 2 minutes. The Dry Tips were detached using fluoride-free water spray around the edges of the pad and removed. The Dry Tips were weighed again to measure the quantity of collected saliva.

Figure 4.2 shows the collection of ductal saliva using Dry Tips in an adult volunteer. Ductal saliva was successfully collected using the Dry Tips and the volume of the collected saliva samples ranged from 1.5 - 5.2 ml.

**Figure 4.1. Commercially available Dry Tips, large size for adults and small size for children.**

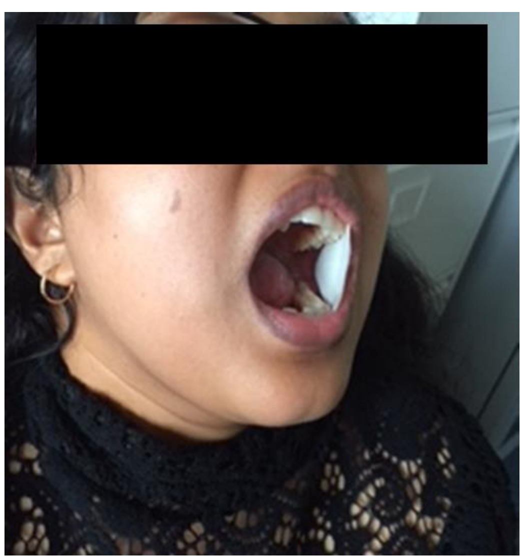

**Figure 4.2. Ductal saliva collection using Dry Tips in an adult volunteer.**

#### 4.3.3.3. Collection of saliva samples using Lashley Cups

i) Manufacturing Lashley Cup

The original plan was to purchase the Lashley cups for this study. However, with no commercially available Lashley cups in the UK or available internationally online, a local hospital (James Cook University Hospital (JCUH)) was contacted to find out if the Lashley cup or any similar device had ever been used by the staff. The technicians and staff approached at various departments at JCUH were not aware of any such device.

The Lashley cup was extensively searched online and a couple of research articles (Percival *et al.,* 1994 and Booth *et al.,* 2007) suggested that it had previously been used by staff at the Medical Physics Department, Guys Hospital, London, as well as the Dental School, Newcastle University. Both possibilities were contacted and no current use or possession of the Lashley cup was obtained.

After being unable to locate the Lashley cup, it was decided to manufacture them at Teesside University. Lashley cups were successfully manufactured by the Department of Engineering at Teesside University using a model from Professor M. Buzalaf (São Paulo University) (Figure 4.3). Although the model was manufactured using stainless steel, the Lashley cups made for this project used polytetrafluoroethylene (PTFE) which is a highly inert and non-toxic material often used in medical devices (Drobny, 2006). It is also suitable for autoclaving to ensure maximum sterility and this was tested by the Dental Team at Teesside University where all components of the Lashley cup including collection tubes, suction tubes and pump (Figure 4.4) were all successfully autoclaved using a vacuum autoclave.

**Figure 4.3. The Lashley cup model.**

**Figure 4.4. Lashley Cup manufactured for this study.** Lashley cup with all its components including collection tubes and pump.

ii) Collection of Ductal saliva

As not being a qualified dental professional, the researcher (Maria Sajjad) was unable to perform the saliva collection due to the ethical guidelines. Therefore, the Lashley cups manufactured at Teesside University were tested at three different levels on five participants in total:

1) They were taken to Brazil by Dr Liane Azevedo (Co-supervisor) who was visiting São Paulo University for another project. A dentist experienced in collecting ductal saliva at Bauru, Brazil tested the Lashley cups on two volunteers: one adult (Dr Azevedo) and one child (a colleague's child). The dentist reported to successfully having collected parotid

ductal saliva using the Lashley cup manufactured at Teesside University. The use of the manufactured Lashley cup is demonstrated by Figure 4.5.

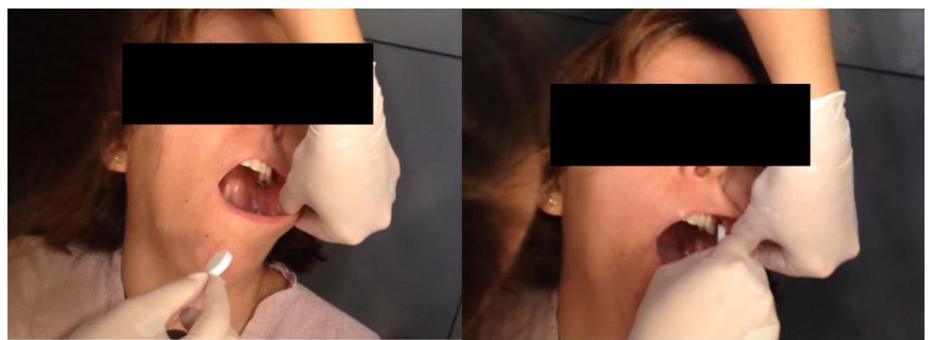

**Figure 4.5. Lashley cup testing in adult (above) and child (below) volunteer.**

2) A dental therapist from the Dental School at Teesside University, Middlesbrough, was shown two video clips demonstrating how to use the Lashley cup. She tested the Lashley cup on one adult volunteer (Professor Vida Zohoori (Supervisor)).

3) An experienced dental nurse from the Dental Hospital, Newcastle University, Newcastle, was also shown two video clips demonstrating how to use the Lashley Cup. She also tested the Lashley cup on two adult volunteers (Professor Vida Zohoori (Supervisor) and another dental nurse from the same department).

Before sampling, the mouth was rinsed using 100 ml deionised water and the inner cheek (surrounding buccal mucosa) was cleaned with a gauze moistened with deionized water.

Lashley cups were placed over the Stephenson's duct of the left or right cheek and attached using suction by a hand bulb vacuum pump. The salivary flow rate was stimulated using a few drops of lemon juice with a swab wiped on the tongue at 30 second intervals for 2 minutes. A minimum of 2 ml saliva was aimed to be collected for fluoride analysis. The Lashley cups was then gently removed after decreasing suction from the pump.

### 4.3.4. Data Handling and Analysis

#### 4.3.4.1. Fluoride content of Dry Tips

1) Ashed

- Fluoride content (μg/DT): The fluoride content of ashed Dry Tips (μg/ashed DT) was calculated by multiplying the fluoride concentration of the ashed sample (μg/g) by the weight of ash (μg).
- Percentage weight loss (%): The percentage weight loss of ashed samples was calculated using the following formula:

  Weight loss (%) = [(original weight of Dry Tip with sample (g) – final weight of ash (g)) ÷ original weight of Dry Tip with sample (g)] x 100.

- Percentage recovery of added fluoride (%): The percentage recovery of added fluoride to the Dry Tips prior to ashing was calculated using the following formula:

  Fluoride recovery (%) = [measured fluoride content (μg/DT) of spiked Dry Tip sample ÷ theoretical fluoride content of Dry Tip (μg/DT)] x 100.

2) Un-ashed

- Fluoride content (μg/DT): The fluoride concentration of Dry Tips (μg/g) was multiplied by the weight of Dry Tips (g) to calculate the fluoride content of each Dry Tip (μg/DT).
- Percentage Recovery of added fluoride (%): The percentage recovery of added fluoride to the Dry Tips was calculated using the following formula:

Fluoride recovery (%) = [measured fluoride content (μg/DT) of spiked Dry Tip sample ÷ theoretical fluoride content of Dry Tip (μg/DT)] x 100.

#### 4.3.4.2. Statistical Analysis

Descriptive analysis was used to calculate mean and standard deviations for the Dry Tip samples using Microsoft Excel 2016.

## 4.4. Results

### 4.4.1. Dry Tips

#### 4.4.1.1. Pre-preparation Technique

The Dry Tips were successfully ashed using the muffle furnace. The colour and consistency of the ashed Dry Tips varied across the various samples that were ashed. Table 4.1 displays images of all un-ashed and ashed Dry Tip samples using ceramic and nickel crucibles. Using the ceramic crucibles, the appearance of the resultant ash of blank Dry Tips was dark grey with a light fluffy texture. The appearance became lighter and texture denser as the concentration of added fluoride standards increased.

The appearance of the resultant ash of blank Dry Tips using nickel crucibles was light grey and soft. The colour and texture of the ash became darker and crispier as the concentration of added fluoride standards increased.

The ashing of the Dry Tip samples with ashing aid was not as successful as those without ashing aid. The texture of the resultant sample was solid, dried salt instead of soft ash. The ashed sample was dissolved in dd$H_2O$ to remove from the crucible, ready for fluoride analysis.

**Table 4.1 Images of un-ashed and ashed Dry Tip samples.**

| Sample | Un-ashed | Ceramic Crucible Ashing | Nickel Crucible Ashing |
|---|---|---|---|
| Blank | | | |
| With added $ddH_2O$ | | | |
| With added 0.1 ppm F standard | | | |
| With added 0.5 ppm F standard | | | |
| With added 1 ppm F standard | | | |
| With added 1 ppm F standard with NaOH | | | *N/A |

Note: *Not Available (N/A).

The weight of the Dry Tips before ashing ranged from 0.79 to 0.89 g. The successful ashing process of the Dry Tip samples without ashing aid using both ceramic and nickel crucibles was supported by the average percentage weight loss as presented in Table 4.2. The average percentage weight loss for ceramic crucibles ranged from 60.4% to 98.0% compared to nickel crucibles ranging from 87.0% to 95.9%.

The ashing of the Dry Tip samples with ashing aid was not as successful as those without ashing aid. The percentage weight loss of the Dry Tip with ashing aid was 60.4% (Table 4.2) and it was very difficult to remove the sample from the crucible due to the formation of dried salt which stuck to the bottom of the crucible, hence requiring to be dissolved in dd$H_2O$ to remove from the crucible.

**Table 4.2. Average percentage weight loss of Dry Tip samples ashed using Nickel and Ceramic Crucibles.**

| Dry Tip (DT) | Average weight loss (%) | |
|---|---|---|
| | Nickel Crucible | Ceramic Crucible |
| **Blank** | 87.9 (n= 13) | 94.7 (n= 6) |
| **DT with dd$H_2O$** | 95.7 (n= 13) | 97.8 (n= 6) |
| **DT with 0.1 ppm F standard** | 93.6 (n= 4) | *N/A |
| **DT with 0.5 ppm F standard** | 87.0 (n= 8) | 97.6 (n= 3) |
| **DT with 1 ppm F standard** | 95.9 (n= 5) | 98.0 (n= 6) |
| **DT with 1 ppm F standard and NaOH as ashing aid** | *N/A | 60.4 (n= 3) |

Note: *Not Available (N/A).

### 4.4.1.2. Endogenous fluoride content of Dry Tips

Fairly high concentrations of fluoride were detected from the blank Dry Tips, regardless of the method used (Table 4.3). The results were inconsistent and therefore unreliable across the ashed and un-ashed samples. An average (SD) fluoride concentration of 0.089 (0.117) µg/g was detected in the ashed Dry Tips using ceramic crucibles compared to the relatively higher average (SD) fluoride concentration of 1.351 (2.121) µg/g found in Dry Tips using nickel crucibles.

On the other hand, the un-ashed blank Dry Tips yielded an average (SD) fluoride concentration of 0.360 (0.693) µg/g per whole and 0.354 (0.273) µg/g per cut Dry Tip samples.

**Table 4.3. Mean (SD) endogenous fluoride concentrations of Blank Dry Tips by preparation method.**

| Preparation Method | Original Dry Tip weight (g) | Fluoride Concentration (µg/g) | Fluoride Content (µg/DT) |
|---|---|---|---|
| **Ashed:** | | | |
| Ceramic (n= 6) | 0.828 (0.010) | 0.089 (0.117) | 0.074 (0.097) |
| Nickel (n= 13) | 0.814 (0.018) | 1.351 (2.121) | 1.106 (1.748) |
| **Un-ashed:** | | | |
| Whole (n= 17) | 0.735 (0.152) | 0.360 (0.693) | 0.253 (0.503) |
| Cut (n= 6) | 0.510 (0.006) | 0.354 (0.273) | 0.180 (0.139) |

Note: * Child size Dry Tips were used for the un-ashed cut method. The remaining methods used adult sized Dry Tips.

### 4.4.1.3. Recovery of added fluoride

1) Ashed

Table 4.4 highlights the mean (SD) fluoride content of the spiked Dry Tip samples and their percentage recovery using ceramic and nickel crucibles. Varying amounts of fluoride was detected from the Dry Tip samples using both ceramic and nickel crucibles and added fluoride was not recovered successfully. The mean (SD) percentage recovery of fluoride from the spiked samples ranged from 0.1 (0.1)% to 43.1 (30.4)% for ceramic crucibles and from 0.8 (1.0)% to 93.0 (70.7)% for nickel crucibles.

The theoretical fluoride content of spiked Dry Tip samples was estimated using the mean fluoride content of blank Dry Tips i.e. 0.074 (µg/DT) and 1.106 (µg/DT) for ceramic and nickel crucibles, respectively, followed by adding the known content of the added fluoride standards.

**Table 4.4. Mean (SD) fluoride content of spiked Dry Tip (DT) samples and their percentage recovery using ceramic and nickel crucibles.**

| Dry Tip (DT) sample | Mean (SD) | | | | | |
|---|---|---|---|---|---|---|
| | Ceramic Crucibles | | | Nickel Crucibles | | |
| | Fluoride content (µg/DT) of spiked samples | | % recovery | Fluoride content (µg/DT) of spiked samples | | % recovery |
| | Measured | Theoretical** | | Measured | Theoretical** | |
| **DT with $ddH_2O$** | 0.03 (0.02) | 0.07 | 43.1 (30.4) | 1.77 (3.83) | 1.11 | 21.9 (29.6) |
| **DT with 0.1 ppm F standard** | *N/A | *N/A | *N/A | 0.01 (0.01) | 1.21 | 0.8 (1.0) |
| **DT with 0.5 ppm F standard** | 0.08 (0.07) | 0.57 | 14.5 (11.5) | 2.72 (2.52) | 1.61 | 93.0 (70.7) |
| **DT with 1ppm F standard** | 0.27 (0.29) | 1.07 | 25.1 (27.2) | 0.18 (0.21) | 2.11 | 8.6 (10.1) |
| **DT with 1ppm F standard with ashing aid** | 0.00 (0.00) | 1.07 | 0.1 (0.1) | *N/A | *N/A | *N/A |

Note:

* Not Available (N/A).

** Calculated using the mean fluoride content of blank Dry Tips i.e. 0.074 (µg/DT) for ceramic and 1.106 (µg/DT) for nickel crucibles followed by adding the known content of the added fluoride standards. Standard deviation was not reported as the theoretical values were estimated to gain a single value with no variation.

2) Un-ashed

The mean (SD) fluoride content of the spiked cut and whole Dry Tip samples and their percentage recovery are displayed in Table 4.5. Fluoride content of the un-ashed samples also varied across cut and whole Dry Tip samples. The mean (SD) percentage recovery of fluoride from the spiked samples ranged from 57.4 (126.7) % to 264.5 (31.9) % for whole Dry Tips and from 49.9 (31.9) % to 109.8 (63.7) % for cut Dry Tips.

The theoretical fluoride content of spiked Dry Tip samples was estimated using the mean fluoride content of blank Dry Tips i.e. 0.253 (µg/DT) and 0.180 (µg/DT) for whole and cut Dry Tips, respectively, followed by adding the known content of the added fluoride standards.

**Table 4.5. Mean (SD) fluoride content of spiked Dry Tip (DT) samples and their percentage recovery using Whole and Cut Dry Tips.**

| Dry Tip (DT) sample | Mean (SD) | | | | | |
|---|---|---|---|---|---|---|
| | Whole | | | Cut | | |
| | Fluoride content (µg/DT) of spiked samples | | % recovery | Fluoride content (µg/DT) of spiked samples | | % recovery |
| | Measured | Theoretical** | | Measured | Theoretical** | |
| **DT with $ddH_2O$** | 0.146 (0.322) | 0.254 | 57.4 (126.7) | 0.116 (0.102) | 0.180 | 64.7 (56.6) |
| **DT with 0.1 ppm F standard** | 0.821 (0.818) | 0.411 | 224.5 (231.1) | 0.140 (0.089) | 0.280 | 49.9 (31.9) |
| **DT with 0.5 ppm F standard** | 0.468 (0.220) | 0.754 | 62.1 (29.2) | *N/A | *N/A | *N/A |
| **DT with 1ppm F standard** | 1.775 (1.077) | 1.254 | 141.6 (85.9) | 1.296 (0.751) | 1.180 | 109.8 (63.7) |
| **DT with 10 ppm F standard** | 27.124 (3.271) | 10.254 | 264.5 (31.9) | *N/A | *N/A | *N/A |

Note:

* Not Available (N/A).

** Calculated using the mean fluoride content of Dry Tips i.e. 0.253 (µg/DT) for whole and 0.180 (µg/DT) for cut, followed by adding the known content of the added fluoride standards. Standard deviation was not reported as the theoretical values were estimated to gain a single value with no variation.

### 4.4.2. Lashley Cups

The Lashley cup was successfully manufactured at Teesside University using the model from Buzalaf, Brazil. This section highlights the successes and challenges in collection of ductal saliva using the produced Lashley cups.

#### 4.4.2.1. Collection by an experienced dentist

The Teesside University manufactured Lashley cup was tested in Brazil to check its performance by a qualified dentist with extensive experience of using Lashley cups in Brazil. The dentist in Brazil performing the saliva collection reported successful saliva collection using the Lashley cup produced at Teesside University. However, the collected samples were unable to be transported to the UK for fluoride analysis.

#### 4.4.2.2. Collection by a qualified dental therapist without prior experience of collecting ductal saliva

There were many challenges faced in the collection of ductal saliva by the dental therapists at Teesside University. Several attempts were made by the dental therapists to locate the duct and collect saliva. Due to the collection being unsuccessful, other trained and qualified dental colleagues were approached to help with the collection. Despite spending more than one hour with the volunteer, the dental team were not able to collect any ductal saliva samples (due to being unable to locate the duct), using the Lashley cup, from the volunteer.

#### 4.4.2.3. Collection by qualified dental research nurses without prior experience of collecting ductal saliva

The dental nurses at Newcastle Dental Hospital also faced challenges during the collection of ductal saliva. These nurses were fully qualified with more than ten years of research and clinical experience at Newcastle University. Four attempts were made on two different volunteers. However, again, no sample was collected, again due to difficulty in locating the parotid duct.

## 4.5. Discussion

### 4.5.1. Dry Tips

To release fluoride from its matrix, different methods of preparation such as ashing, fusion with an alkali, digestion with an acid or HMDS micro-diffusion have been suggested for different samples (Tylenda, 2003). Whitford *et al.* (1999) used ashing as a method to remove the organic material from finger nails before fluoride analysis. The use of NaOH has also been suggested as an ashing aid for determination of fluoride in biological samples (Malde *et al.*, 2001).

For this preliminary study, the technique of open ashing, with and without ashing aid, and HMDS acid diffusion method were used to prepare the Dry Tip samples for fluoride analysis. The ashing protocol was adapted from Whitford *et al.,* (1999) which used porcelain crucibles to ash the samples in a muffle furnace at 600°C.

Following the method by Malde *et al.* (2001), ashing aid (NaOH) was added to a set of samples prior to ashing (Table 4.4). This was included in the protocol to prepare the samples for ashing in order to eliminate any potential loss of fluoride during the ashing process that may have occurred. However, this failed to work as no fluoride was recovered from the sample that used NaOH as an ashing aid. This indicated the need to review the technique being used, upon which the Malde group from Norway was contacted to share the detailed protocol for the method used in their study. The detailed methodology was written in Norweign but Malde kindly offered to send copies of her thesis which included the discussion around the method used. After carefully studying Malde’s thesis which discussed the suitability of NaOH as an ashing aid for food samples, it was decided that NaOH as an ashing aid may not be suitable for Dry Tips.

The method by Malde suggested using nickel crucibles; whereas, the protocol set out by Whitford *et al.* (1999) used porcelain crucibles. However, extensive search of the various crucible materials available from manufacturers and their chemical properties suggested incompatibility of porcelain with HF and alkali salts (Tedpella, 2016). Therefore, the instability across the results found may have resulted from the material of crucibles used.

Platinum and nickel have also been recommended as suitable materials for the ashing procedure involving fluoride analysis (Alary *et al.,* 1983 and Thomas *et al.,* 1977).

Furthermore, 'Fisher Scientific Laboratory Supplies' were contacted to request the recommended crucible material for fluoride analysis. Manufacturer recommendation was to use "high purity alumina" for fluoride analysis. Upon searching chemical properties of Alumina, otherwise known as Aluminium Oxide ($Al_2O_3$) Ceramic, it was learnt that '*Alumina resists attack by all gases except wet fluorine and is resistant to all common reagents except hydrofluoric acid and phosphoric acid'* (Accuratus, 2013).

Following the analysis of crucible materials and the incompatibility of the ceramic crucibles that were previously used, the Dry Tips ashing experiment was repeated using nickel crucibles based on the method described by Malde *et al.* (2001). The experiment was repeated to determine the endogenous fluoride content of Dry Tips along with investigating the recovery of added fluoride from the Dry Tips. The resultant ash was darker in colour compared to ash from the ceramic crucibles and its consistency was sponge-like compared to the powder-like consistency of ceramic ash. Dry Tip samples ashed using ceramic crucibles had a mean (SD) fluoride content of 0.074 (0.097) µg/DT compared to a much higher mean (SD) fluoride content found by Dry Tips ashed using nickel crucibles of 1.106 (1.748) µg/DT.

The un-ashed Dry Tips were prepared whole and cut. They were cut in order to increase the surface area of the Dry Tip to come into contact with acid and HMDS to release more fluoride. However, the mean (SD) fluoride content of the whole un-ashed Dry Tips was found to be higher (0.253 µg/DT) than that of the cut Dry Tips (0.180 µg/DT).

Overall, the results from the 'ashed' and 'un-ashed' Dry Tip indicated high endogenous fluoride content of samples. In addition, analysis of different batches of Dry Tips found varying results which indicated that the fluoride content of each Dry Tip varies within each pad and across batches. This may be due to contamination during the manufacturing process or the endogenous fluoride content of materials used to manufacture the Dry Tip which consists of an impermeable plastic film and nylon mesh.

In summary, Dry Tips contain high endogenous fluoride and are unsuitable for the use in studies involving fluoride analysis. The mean fluoride contents of Dry Tips in this study were found to be higher than the fluoride concentration of ductal saliva. The concentration of ductal saliva is known to range from 3.23 - 284 ng/ml (Oliveby *et al.,* 1989; Yao and Gron, 1970; and Table 2.4), whereas the concentration of Dry Tips ranged from 89 - 1351 ng/ml (equivalent to 0.089 - 1.351 µg/g). Moreover, added fluoride was unable to be recovered from the Dry Tips during this investigation which means fluoride from saliva samples would

also be unlikely to be recovered from Dry Tips. The volunteers also reported discomfort whilst the Dry Tips were removed from their inner cheek suggesting it may not be a widely accepted collection method amongst participants, especially younger participants i.e. children.

### 4.5.2. Lashley Cups

A Lashley cup was successfully manufactured in the UK using the model from Buzalaf. The main advantages of the manufactured Lashley cup arose from the non-toxic and inert properties of the material used for manufacture i.e. polytetrafluoroethylene (PTFE). This allows the Lashley cup to be autoclaved after each use for maximum sterility. The Lashley cup with all its components was tested in a vacuum autoclave and was successfully autoclaved which allow each Lashley cup to be used several times. Thus, increasing the cost effectiveness of the product and its manufacturing costs. However, the ultimate disadvantage arose from its failure in collection of ductal saliva by non-experts. Since specialist training is required to correctly identify the parotid duct, inaccurately locating the parotid duct may have been the main reason for no saliva collection by the dental nurses and therapists who were involved with the Lashley cup testing in the UK.

Therefore, dental professionals with greater experience and specialist skills are more suited to using the Lashley cup. In summary, considering the implications in locating the parotid duct, the use of Lashley cups may not be favourable in the wider population, due to individual anatomical variations of the mouth as well as the need for a trained dentist to perform the sampling.

### 4.5.3. Other methods of ductal saliva collection

Due to unsuccessful collection of ductal saliva using the Lashley cups, alternative methods for ductal saliva collection were considered. Use of a portable extraction pump that is commonly used within dental practices could be an alternative method. However, sterilisation of the tools and collection of the sample without contamination would be the greatest challenge. Therefore, the feasibility and practicality of such a method was not ideal.

The collection of sublingual-submandibular (SL-SM) saliva as a measure of recent fluoride exposure was then considered due its direct relation with plasma. A ratio of 0.55 ± 0.13 and

0.69 ± 0.11 has been found between plasma and unstimulated and stimulated SL-SM saliva, respectively (Oliveby *et al.,* 1989). Wolff *et al.* (1997) developed a novel system to collect SL-SM saliva consisting of a four parts-collecting tubing, a buffering chamber, a storing tube, and a suction device. They collected stimulated and unstimulated SL-SM saliva for 1 minute from ten healthy and ten xerostomic individuals with mean flow rates of 0.165 ± 0.073 ml/min/gland and 0.024 ± 0.013 ml/min/gland for unstimulated saliva, respectively. The mean flow rate for stimulated saliva was reported as 0.493 ± 0.013 ml/min/gland, however, these samples were not analysed for fluoride. Oliveby *et al.* (1989) investigated the fluoride levels in SL-SM saliva by using a collection device made from silicone impression material with a plastic tubing to collect the saliva from the ducts of the submandibular gland in ten-minute fractions. They found fluoride concentrations of 0.48 ± 0.19 µmol/l and 0.44 ± 0.25 µmol/l for stimulated and unstimulated SL-SM saliva, respectively and due to the direct relation of SL-SM with plasma, the collection of SL-SM saliva was considered for future work.

## 4.6. Conclusion

### 4.6.1. Dry Tips

Results from this investigation of Dry Tips found the endogenous fluoride content of Dry Tips being higher than the fluoride concentration of ductal saliva. Therefore, they are unsuitable for the use of ductal saliva collection for fluoride investigation in this study.

### 4.6.2. Lashley cups

The challenges in terms of practicality and feasibility of using Lashley cups with the wider population including children as well as the requirement of a trained dentist to perform the collection, led to the conclusion of ruling out the use of Lashley cups for the collection of ductal saliva in this study.

## 4.7. Recommendations for Future Studies

Further work is required to develop the use of Lashley cups for ductal saliva collection in a separate study. The design of the apparatus may need to be reconsidered to make it easier for the dental professionals to be able to locate and fit the Lashley cup over the parotid duct.

Investigation into the collection of SL-SM saliva as an appropriate alternative to parotid ductal saliva is also required.

# Chapter 5. Study 2: Determination of fluoride in saliva and plasma: sample collection and analysis.

## 5.1. Introduction

Fasting plasma fluoride levels are in equilibrium with bone fluoride in the human body (Parkins *et al.,* 1974). This direct relation between bone and plasma fluoride allows plasma to be used as a useful indicator of bone fluoride levels (Carvalho *et al.,* 2006) and thus, gain a better understanding of fluoride metabolism.

However, the collection of blood plasma through venous sampling can be invasive, unethical and poses the risk of harm. This can be a major drawback, especially when involving young children. Therefore, there is need for the search of other bodily fluids which have a direct relation with plasma and can be collected in a simple, practical and non-invasive manner.

Previous studies have demonstrated a proportional relation between the fluoride concentrations in plasma and parotid ductal saliva in children (Whitford *et al.,* 1999; Fukushima *et al.,* 2011); and in adults (Ekstrand, 1977[A]; Whitford, 1996 and Fukushima *et al.,* 2011); as well as sublingual-submandibular saliva in children (Twetman *et al.,* 1998); and adults (Whitford, 1996). All, which can be collected non-invasively.

Fluoride concentrations in plasma are on average slightly higher than parotid and sublingual saliva. However, they all correlate proportionally with a constant ratio of 0.80 between parotid and plasma fluoride (Whitford *et al.,* 1999) and 0.55 and 0.69 between unstimulated and stimulated sublingual-submandibular and plasma fluoride, respectively (Oliveby *et al*., 1989). These findings suggest that the fluoride concentrations in ductal saliva can be determined by the simultaneous concentrations in plasma.

Therefore, this study aimed to: 1) collect blood samples and investigate the association between fluoride concentrations in whole blood and plasma (Study 2A); and 2) collect blood and saliva samples and investigate the relation between fluoride concentrations in blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva (Study 2B).

# Study 2A: Association between fluoride concentrations in whole blood and plasma.

## 5.2. Aims and objectives

The main aim of this study was to investigate the association between fluoride concentrations in whole blood and plasma.

**Subsidiary Aims**

- To investigate the practicality of venous and capillary blood sampling.

**Objectives**

- To investigate the practicality of venous blood sampling by venepuncture.
- To investigate the practicality of capillary blood sampling by dermal puncture of fingertip (finger prick).
- To measure fluoride concentrations of whole blood samples collected by 1) venous and 2) capillary (finger prick) blood sampling.
- To compare fluoride concentrations of venous and capillary whole blood.
- To measure fluoride concentrations of plasma samples collected using venous blood sampling.
- To compare fluoride concentrations of whole blood and plasma collected using venous blood sampling.

## 5.3. Materials and Method

### 5.3.1. Ethical Approval

This project was approved by the Research Governance and Ethics Committee, School of Health and Social Care, Teesside University. Reference number 066/15 (approval attached – Appendix 1). The complete ethics application with supporting documents are also attached (Appendix 2).

### 5.3.2. Study Location

These studies took place at Teesside University, located in Middlesbrough situated in the north east of England. Middlesbrough is a non-fluoridated area, with a water fluoride concentration of <0.3 ppm.

### 5.3.3. Participants

Participants were healthy adults living in non-fluoridated areas from the Teesside area. Both male and female participants were recruited. All healthy adults aged 19 - 45 years were eligible to participate, unless they:

- Had been diagnosed with a dry mouth condition.
- Were pregnant.
- Had recent oral surgery.
- Had current oral disease or infection.
- Had gum disease.

### 5.3.4. Sample size

A convenient sample size of around 10 was decided after reviewing a paper by Cardoso *et al*. (2008) who investigated the daily variations in human plasma fluoride concentrations using 5 adults (aged 27 to 33 years).

### 5.3.5. Recruitment

Recruitment material was circulated amongst staff and students across the five schools/departments of Teesside University; i.e. an approximate 2,396 staff and 18,576 students (full time and part time) were contacted. Invitations were also open to family and friends of staff and students at Teesside University.

An invitation email with the participant information sheet attached was circulated by the researcher (Maria Sajjad) via the school administrator. An item was also displayed in the Health and Social Care Institute newsletter to advertise the study and invite participants. Those participants interested in taking part contacted the researcher by

telephone or email. It was firstly ensured that participants were eligible and free from the exclusion criteria. Participants were then invited to attend a meeting immediately prior to data collection where the nature and design of the study was discussed, and participants were given the opportunity to ask any questions or concerns they had regarding the research before signed consent was obtained.

Participants were given a £15 'Love 2 Shop' gift voucher at the end of the study, to thank them for their time in sample collection.

## 5.3.6. Sample collection and preparation

The blood samples were collected in non-fasting condition, following the participant's usual diet and routine of tooth brushing.

### 5.3.6.1 Venous Whole Blood

A trained and qualified nurse collected 10 ml venous whole blood from a vein in the antecubital fossa of either left or right arm. The blood samples were drawn into Vacutainer® spray-coated $K_2$ EDTA blood collection tubes (BD, Oakville) and inverted several times before storage in a refrigerator. A 3 ml aliquot was taken for fluoride analysis of whole blood (in triplicate i.e. 3 x 1 ml) and the remaining was used to separate venous plasma (section 5.3.6.2).

### 5.3.6.2 Venous Plasma

The remaining 7 ml venous whole blood collected in section 5.3.6.1, was centrifuged at 1500 rpm for 10 minutes to separate the plasma. Around 3 – 4 ml plasma was separated, and the remaining blood cells were disposed safely in a yellow bio-hazardous waste bin. The separated plasma was transferred into sterile bijous tubes for fluoride analysis (in triplicate i.e. 3 x 1 ml) and stored in a freezer at -18ºC until analysis.

### 5.3.6.3 Capillary whole blood

A lancing device with sterile lancets was used to prick the index finger of each human participant. The finger from the same arm as venous blood sampling was used. Around 7-10 drops of capillary whole blood were collected by gently and firmly milking the finger till

blood flow discontinued. The sample was carefully drawn using a pipette into a pre-weighed petri dish and refrigerated immediately for fluoride analysis.

### 5.3.7 Sample analysis

The blood and plasma samples were analysed using the HMDS acid-diffusion method – as described in chapter 4 (Martinez-Mier *et al.,* 2011).

### 5.3.8 Data Handling and analysis

**Ratio:** Ratio between whole blood and venous plasma was calculated by (dividing the fluoride concentration of whole blood (µmol/l) by fluoride concentration of venous plasma (µmol/l)).

Descriptive statistics was performed using statistical software, IBM SPSS Statistics (version 24) and Microsoft Excel 2016. Mean and standard deviations were calculated. A paired t-test was conducted to compare mean differences between blood samples. Regression analysis and Pearson correlation were performed to examine the associations between fluoride concentrations in:

- Venous whole blood and capillary whole blood.
- Capillary whole blood and venous plasma.
- Venous whole blood and venous plasma.

## 5.4. Results

### 5.4.1. Subject

Recruitment for study 2A was undertaken between October 2015 and March 2016. Of those contacted, 16 people were interested and consented to take part. However, one (male) participant had to be excluded from the study as no blood was obtained following two attempts of venepuncture and according to the Teesside University guidelines on blood collection, it is not permissible to insert a needle more than twice in the same individual in one week. In total, 15 participants took part in study 2A, 10 males and 5 females, aged 19 - 35 years (mean age, 28.6 years).

### 5.4.2. Fluoride concentration of whole blood and plasma

Table 5.1 presents the fluoride concentrations in whole blood and plasma samples, along with the mean (95% CI) difference in blood and plasma samples. The fluoride concentration in capillary whole blood (133.1 ng/ml) was much higher than venous whole blood (8.8 ng/ml) and venous plasma (13.5 ng/ml). There was a significant mean difference (-124.3 ng/ml, p = 0.002) between venous and capillary whole blood.

On the other hand, there was a mean difference of 4.8 ng/ml (p = 0.001) between venous whole blood and venous plasma.

**Table 5.1. Mean (SD) fluoride concentrations in whole blood and plasma samples with the mean (95% Confidence Interval (CI) difference between whole blood and plasma. Results are reported as ng/ml as well as *µmol/l*, (n= 15).**

| Units | Whole blood | | Plasma | Mean (95% CI) difference between | |
|---|---|---|---|---|---|
| | Venous | Capillary | Venous | Venous and Capillary whole blood | Venous whole blood and Venous plasma |
| ng/ml | 8.8 (1.4) | 133.1 (125.1) | 13.5 (0.7) | -124.3 (-193.7, -54.9) | 4.8 (2.3, 7.2) |
| *µmol/l* | *0.46 (0.07)* | *7.01 (6.58)* | *0.71 (0.27)* | *-6.29 (-10.00, -2.59)* | *-0.25 (-0.38, -0.12)* |

The mean (SD) fluoride concentration in capillary whole blood was much greater (133.1 (125.1) ng/ml) than the mean fluoride concentration in venous whole blood (8.8 (1.4) ng/ml). Figure 5.1 illustrates the relationship between fluoride concentrations in capillary whole blood and venous whole blood samples. There was no significant relation between fluoride concentrations in capillary and venous whole blood (Pearson correlation = 0.528, p = 0.117).

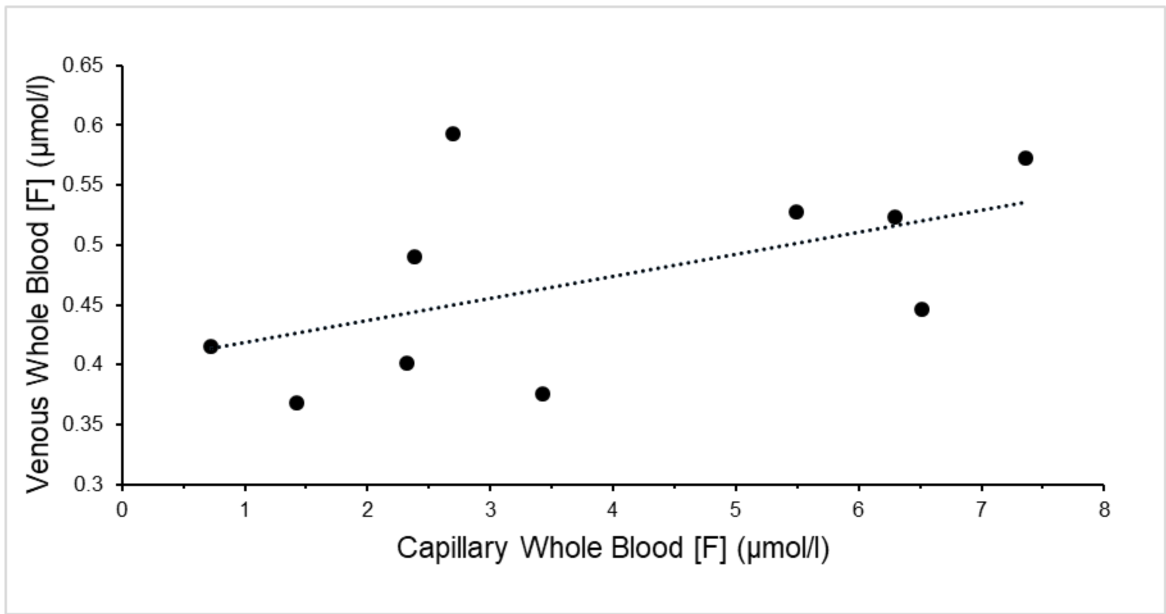


**Figure 5.1. Relation between fluoride concentrations in venous whole blood (VWB) and capillary whole blood (CWB) (n= 10). VWB= 0.401 + (0.018 x CWB). (Pearson correlation = 0.528, p = 0.117).**

Figure 5.2 illustrates the relation between fluoride concentrations in capillary whole blood and venous plasma. A non-significant negative correlation was found (Pearson correlation = -0.009, p value = 0.981).

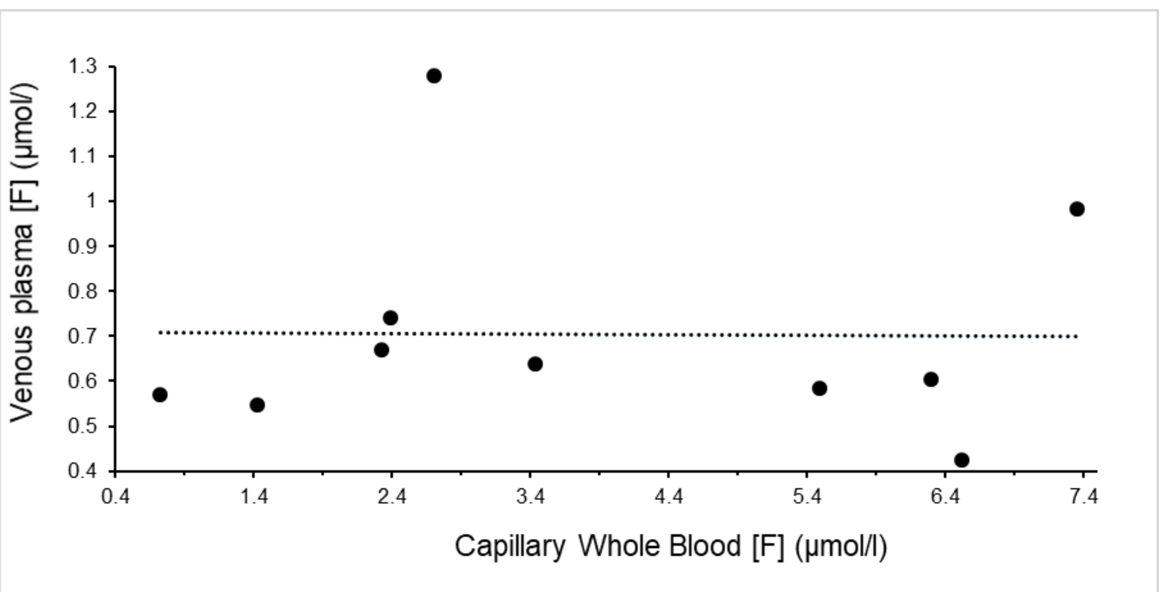


**Figure 5.2. Relation between fluoride concentrations in capillary whole blood (CWB) and venous plasma (VP). VP = 0.708 + (-0.009 x CWB). (Pearson correlation = -0.009, p = 0.981).**

Figure 5.3 illustrates the fluoride concentrations in venous whole blood and venous plasma. A significant positive correlation was found (Pearson correlation = 0.692, p value = 0.027) with mean (SD) fluoride concentrations of 8.6 (1.4) ng/ml and 13.5 (0.7) ng/ml in venous whole blood and venous plasma, respectively.

The mean (SD) ratio between venous whole blood and venous plasma was 0.65 (0.18) ng/ml.

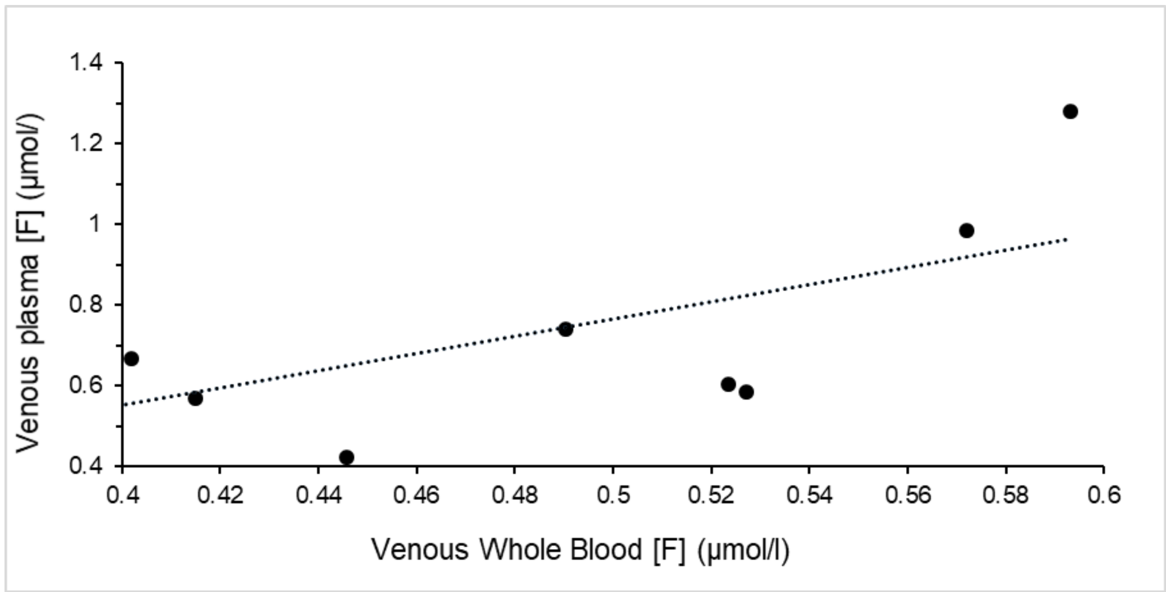


**Figure 5.3. Relation between fluoride concentrations in venous whole blood (VWB) and venous plasma (VP). VP = 0.299 + (2.128 x VWB). (Pearson correlation = 0.692, p = 0.027).**

## 5.5. Discussion

The recruitment rate for this study was good and more participants (n= 16) than anticipated (n= 10) were interested and took part, even with blood sampling involved. Of the 16 recruited, 15 participants completed data collection. No one dropped out and only one participant was excluded due to the failure in blood sample collection. The good recruitment rate may have arisen due to the short time commitment and incentive involved. Data collection for the study was completed in under 15 minutes for each participant.

A fully qualified and competent nurse was recruited from within the Health and Social Care Institute and was approved by the University's insurers (UMAL) for taking blood collection for this study. The nurse had limited availability due to work commitments, however, blood samples were obtained from all recruited participants within the time frame the nurse was recruited.

The mean (SD) plasma fluoride concentration found in this study was 0.71 (6.58) µmol/l (equivalent to 13.5 (0.71) ng/ml), (n= 15) which is within the range from 6 ng/ml to 47 ng/ml reported in the literature for adults of a similar age range (Sener *et al.,* (2007); Parkins *et al.* (1974); Cardoso *et al.* (2006); Cardoso *et al.* (2008); Thomas *et al.* (2016); Ekstrand *et al.* (1977)[A]; Ekstrand (1978); Ekstrand *et al.* (1981); Oliveby *et al.* (1989)[1]; Oliveby *et al.* (1989)[2]; Oliveby *et al.* (1989)[3]; Buzalaf *et al.* (2008); Whitford (1996); Whitford *et al.* (2008); Maguire *et al.* (2005)), as described in chapter 2 (section 2.2.3).

The mean plasma fluoride concentration in this study was similar to findings from Ekstrand *et al.* (1981) who found fasting plasma fluoride concentrations of 13.3 ng/ml in female adults aged 27 - 36 years (n= 5). A study by Oliveby *et al.* (1989)[2] also found similar fasting plasma fluoride concentrations to this study of 12.4 ng/ml in adults aged 26 - 38 years (n= 5) (0.2 ppm water fluoride). The age of participants and fluoride levels of the local water supply in the studies by Ekstrand *et al.* (1981) and Oliveby *et al.* (1989)[2], were in the same range as the current study which supports the findings of the current study. However, participants from the current study were not fasting during sample collection, compared to participants from Ekstrand *et al.* (1981) and Oliveby *et al.* (1989)[2] which were in fasting state during the studies.

It is more usual to report fluoride concentrations in plasma rather than whole blood (Buzalaf *et al.,* 2011) but this study decided to investigate whole blood as an alternative method for blood collection. Mean (SD) fluoride concentration in venous whole blood in the current

study was 0.46 (0.07) µmol/l, which was slightly lower than venous plasma (0.71 (6.58) µmol/l). A ratio of 0.65 was found between venous whole blood and venous plasma which is slightly higher but still in line with Buzalaf *et al.,* (2011) who stated that 'blood cells contain about half the fluoride concentration in plasma'.

The significant positive correlation between fluoride concentrations of venous whole blood and venous plasma may have arisen due to the similarity in the biochemical composition of blood and plasma i.e. blood consists of plasma and therefore the fluoride levels of plasma will be reflected in whole blood. Higher concentrations of fluoride in plasma compared to whole blood have most likely arisen due to the unequal distribution of fluoride in blood and plasma. Plasma is known to contain both ionic and non-ionic forms of fluoride in greater concentration than whole blood (Buzalaf *et al.,* 2011). There may have been small amounts of undetected fluoride when analysing whole blood.

Although, whole blood requires to be centrifuged to gain plasma which can be time consuming and requires appropriate equipment / resources, the greatest advantage arises from the ability to store plasma at -18°C for analysis and future work. The pros outweigh the cons of using plasma as a measure of fluoride absorption and therefore, venous plasma is deemed as the most appropriate method of blood collection for studies on fluoride metabolism in humans.

Establishing the ratio of 0.65 between venous whole blood and plasma indicated that collecting whole blood might be a useful method to measure fluoride absorption. Therefore, it was important to also investigate capillary whole blood. Capillary whole blood is the simplest method for blood collection, poses less risk of harm compared to venous blood sampling and does not require trained staff for collection. However, results from the current study showed no correlation between capillary whole blood and venous whole blood (figure 5.1).

The mean fluoride concentration of capillary whole blood in the current study i.e. 133.1 ng/ml, coincide with findings by Ekstrand (1977)[A] who found capillary fluoride plasma concentrations in the range of 10 - 300 ng/ml. The fluoride concentration of capillary whole blood (133.1 ng/ml) was much higher than venous whole blood (8.8 ng/ml) and venous plasma (13.5 ng/ml). Higher concentration of fluoride in capillary whole blood compared to venous whole blood or venous plasma may result from the anatomical functions of the blood vessels. Capillaries are responsible for the diffusion and exchange of nutrients, waste and oxygen with tissues at a cellular level; whereas, veins drain blood from organs and limbs

(Boundless, 2016). Therefore, higher concentrations of fluoride may be present in the capillaries as absorbed fluoride is transported throughout the body to be distributed at a cellular level.

The location of capillary blood sampling from the index finger was close to the finger nail. As fingernail fluoride is mainly deposited through systemic circulation (Sankhala *et al.,* 2014; Whitford *et al.,* 1999), the concentration of fluoride in capillary whole blood from fingers may also be higher due to the deposition of fluoride into finger nails. Any fluoride not taken up by the nails may be recycled back into the circulatory system via capillary whole blood for renal clearance. Fluoride has a high affinity for hard tissue (e.g. nail and bone) and is reversibly bound to apatite and other calcified tissue (O'Mullane *et al.,* 2016). However, this reversible bond means that fluoride clearance from the bone is reflective in the blood and plasma. This may also be the case in fluoride uptake by the nails, where fluoride clearance is reflective in capillary whole blood, resulting in the high fluoride concentrations of capillary whole blood.

According to capillary blood collection guidelines by Niwinski (2009) the maximum amount of blood which can be collected via capillary blood sampling is 500 µl (equivalent to 10 drops). A study by Ekstrand (1977)[A] collected around 1 to 1.5 ml of capillary blood from one finger tip. Ekstrand (1977)[A] stated that approximately 150 µl of sample is enough for fluoride analysis using the fluoride ion selective electrode. In this study, around 7 - 10 drops of blood were successfully collected from most participants before the puncture site started to clot. However, a couple of participants still bled following the collection of 10 drops and in this case slight pressure was applied using cotton swabs to stop the bleeding. No other challenges were faced during the collection of blood samples.

Although, minimal challenges are involved in capillary blood sampling, Niwinski (2009) stated that capillary blood sampling is unsuitable for: 1) severely dehydrated individuals; 2) individuals with poor circulation; 3) tests that require large volumes of blood (e.g. > 500 µl); 4) coagulation studies that require plasma specimens and 5) testing analytes which may differ in capillary blood compared to venous or arterial blood, e.g. glucose, potassium, protein and calcium (Niwinkski, 2009).

A non-significant relation between fluoride concentrations in capillary and venous whole blood and negative correlation between capillary whole blood and venous plasma was found. This may have arisen from the physiological differences in capillaries and veins, as

mentioned above. Therefore, capillary whole blood is considered as an unsuitable biomarker of fluoride absorption.

## 5.6. Summary of Findings and Conclusions

The following conclusions were made from the findings of this study:

- The collection of capillary and venous whole blood is both practical and can be performed with ease using trained staff.
- There was a significant positive correlation (Pearson correlation = 0.692, p = 0.027) between venous whole blood (VWB) and venous plasma (VP), with a VWB/VP ratio of 0.65.
- Venous plasma was found to be the most suitable biomarker for measuring fluoride absorption levels.
- Capillary whole blood is not a suitable indicator of fluoride absorption levels.

# Study 2B: Relation between fluoride concentrations in blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva.

## 5.7. Aims and Objectives

The main aim of this study was to collect blood and saliva samples and investigate the relation between fluoride concentrations in blood plasma, whole saliva and sublingual-submandibular (SL-SM) saliva (Study 2B).

**Objectives**

- To investigate the use of oral syringes in the collection of SL-SM saliva.
- To measure the fluoride concentration of blood plasma samples.
- To measure fluoride concentrations of SL-SM saliva.
- To measure fluoride concentrations of whole saliva.
- To compare fluoride concentrations of SL-SM saliva and whole saliva.
- To compare fluoride concentrations in blood plasma and saliva.

## 5.8. Materials and Method

### 5.8.1. Ethical Approval

This project was approved by the Research Governance and Ethics Committee, School of Health and Social Care, Teesside University. Reference number 066/15 (approval attached – Appendix 1). The complete ethics application with supporting documents are also attached (Appendix 2).

### 5.8.2. Study Location

The study took place at Teesside University, located in Middlesbrough situated in the north east of England. Middlesbrough is a non-fluoridated area, with a water fluoride concentration of <0.3 ppm.

### 5.8.3. Participants and recruitment

Participants were healthy adults living in non-fluoridated areas from the Teesside area. Both male and female participants were recruited. All healthy adults aged 19 - 45 years were eligible to participate, unless they:

- Had been diagnosed with a dry mouth condition.
- Were pregnant.
- Had recent oral surgery.
- Had current oral disease or infection.
- Had gum disease.

The recruitment method was same as study 2A, section 5.3.5.

### 5.8.4. Sample size

A convenient sample size of 20 individuals was decided after reviewing the paper by Whitford *et al.* (1999) who undertook a study to determine fluoride concentrations in whole saliva, parotid ductal saliva and plasma in children by using 17 individuals (aged 5 to 10 years). A study by Oliveby *et al.* (1989) looked into the relation of whole saliva and plasma fluoride levels using only 5 adults (aged 26 - 38 years).

### 5.8.5. Sample collection and preparation

The saliva and blood samples were collected in non-fasting condition, following the participant's usual diet and routine of tooth brushing.

#### 5.8.5.1. Whole saliva

Participants were given an optional private area to collect their whole saliva. They were provided with a graduated plastic cup and asked to either spit or tilt their head forward and drool their unstimulated whole saliva into the cup. They were asked to continue collection of their whole saliva till 5 ml was collected, as marked by the graduations on the cup.

#### 5.8.5.2. Sublingual-Submandibular saliva

Saliva sampling was performed by an experienced research dental nurse from the Dental Hospital, Newcastle University, Newcastle.

Before sampling, the mouth was rinsed using 100 ml deionised water and the inner cheek (surrounding buccal mucosa) was cleaned with a gauze moistened with deionized water and then dried. Dry Tips were placed over the Stephenson's duct of the left and right cheek to absorb the release of parotid ductal saliva, avoiding contamination of SL-SM saliva. Salivary flow was then stimulated using a few drops of lemon juice with a swab wiped on the tongue at 30 second intervals for 2 minutes. The pooled sublingual-submandibular saliva from the base of the mouth under the tongue was collected using a graduated oral syringe until 2 ml of saliva was collected for fluoride analysis. The Dry Tip pads were then removed from the inside of the cheek.

Saliva samples were immediately transferred in sterile bijous tubes and stored in a refrigerator upon collection and analysed within 48 hours of collection.

#### 5.8.5.3. Venous plasma

A trained and qualified nurse collected 5 ml venous whole blood from a vein in the antecubital fossa of either left or right arm. The blood samples were drawn into 5 ml Vacutainer® spray-coated $K_2$ EDTA blood collection tubes (BD, Oakville) and inverted several times before immediate storage in a refrigerator till further use. Blood samples for plasma were centrifuged at 1500 rpm for 10 minutes to separate the plasma. Around 3 ml plasma (supernatant) was separated using a pipette in a fume cupboard and the remaining blood cells / debris were disposed safely into yellow bio-hazardous bins. The 3 ml aliquot was transferred into sterile bijous tubes for fluoride analysis (in triplicate i.e. 3 x 1 ml) and stored in a freezer at -18$^{o}$C until analysis.

### 5.8.6. Sample analysis

**Venous plasma:** The Plasma samples were analysed using the HMDS acid-diffusion method – as described in chapter 4 (Martinez-Mier *et al.,* 2011).

**Saliva:** The saliva samples were analysed using the direct fluoride ion electrode selective method. This method is a validated method previously published in detail by Martinez-Mier *et al.* (2011). A set of standards using 1 ml of 0.01, 0.1, 1, 10 and 100 ppm fluoride (in duplicate) as well as 1 ml of each saliva sample (in duplicate) were set up in bijous tubes. 100 µl of TISAB III was added to each sample and ran for analysis using the Fluoride Ion Selective Electrode (Model Orion 9609BNWP, Thermo Scientific, USA) and meter (Model Orion 720A+, Thermo Electron Corporation, USA).

### 5.8.7. Data Handling and analysis

**Ratio:** Ratio between SL-SM saliva and venous plasma was calculated by (dividing the fluoride concentration of SL-SM saliva (µmol/l) by fluoride concentration of venous plasma (µmol/l)).

**Prediction of fluoride concentration of Plasma (µmol/l):** Plasma fluoride concentration (µmol/l) was predicted by dividing the SL-SM saliva fluoride concentration (µmol/l) by the mean ratio of SL-SM saliva / venous plasma, calculated as above.

Descriptive statistics was performed using statistical software, IBM SPSS Statistics (version 24) and Microsoft Excel 2016. Mean and standard deviations were calculated. A paired t-test was conducted to compare mean differences between plasma and saliva samples. Regression analysis and Pearson correlation were performed to examine associations between fluoride concentrations in:

- Whole saliva and SL-SM saliva.
- Venous plasma and SL-SM saliva.
- Venous plasma and whole saliva.
- Venous plasma and predicted venous plasma, using whole saliva / venous plasma ratio.
- Venous plasma and predicted venous plasma, using SL-SM saliva / venous plasma ratio.

Microsoft excel was also used to construct graphs to illustrate the above relations.

## 5.9. Results

### 5.9.1. Subject

Overall, 24 participants (9 males and 15 females, aged 18 – 45, mean age 29.2 years) were interested and took part.

### 5.9.2. Fluoride concentration of saliva and plasma

Table 5.2 presents the mean fluoride concentrations found in whole saliva and SL-SM saliva. Mean fluoride concentrations in whole saliva (6.32 μmol/l) were much higher than SL-SM saliva (0.87 μmol/l) with a mean difference of 5.65 μmol/l between whole and SL-SM saliva.

**Table 5.2. Mean (SD) fluoride concentrations in saliva and blood plasma samples, with the mean (95% Confidence Interval (CI) difference between plasma and saliva samples, reported in ng/ml as well as μmol.**

| | Saliva | | Venous Blood | Mean (95% CI) difference between | | |
|---|---|---|---|---|---|---|
| Units | **Whole** | **SL-SM** | **Plasma** | **Whole and SL-SM saliva** | **Whole saliva and Venous plasma** | **Venous plasma and SL-SM saliva** |
| ng/ml | 120.2 (142.3) | 16.5 (16.9) | 116.7 (78.3) | 103.5 (43.2, 163.9) | 0.4 (-70.2, 71.0) | 100.2 (65.3, 135.1) |
| μmol/l | 6.32 (7.49) | 0.87 (0.89) | 6.14 (4.12) | 5.65 (2.75, 8.55) | 0.02 (-3.70, 3.74) | 5.27 (3.44, 7.11) |

### 5.9.3. Comparison between saliva and blood

A slight statistically significant positive correlation was found between fluoride concentrations in whole saliva and SL-SM saliva (Pearson correlation = 0.410, p = 0.05), as illustrated by Figure 5.4.

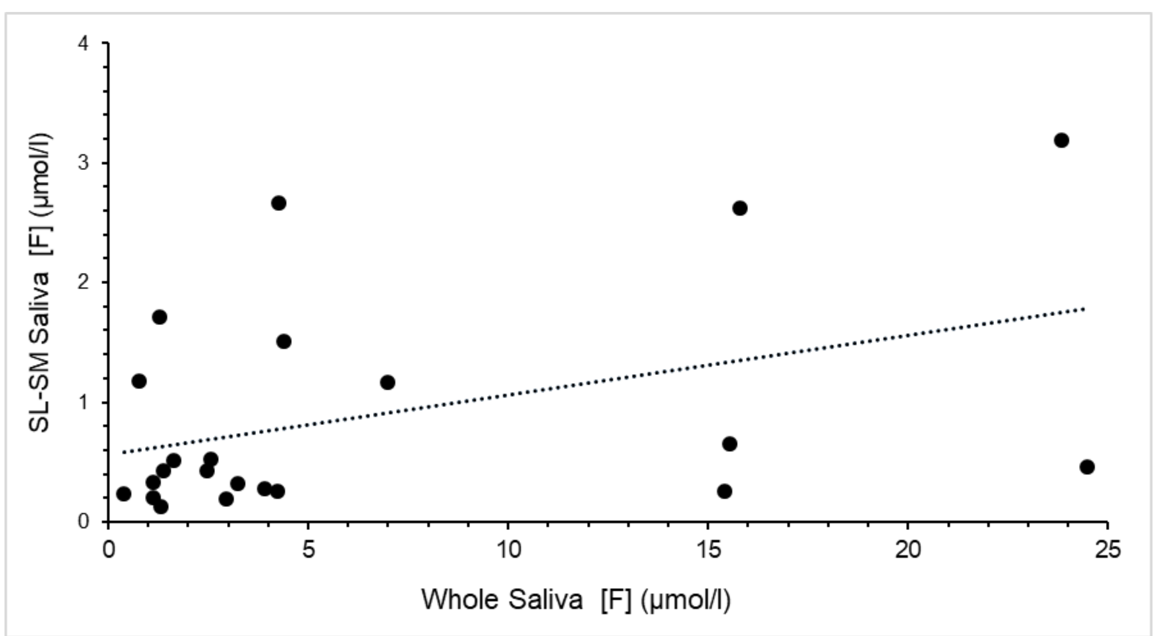


**Figure 5.4. Relation between fluoride concentrations in whole saliva (WS) and SL-SM saliva. SL-SM = 0.5611 + (0.0497 x WS). (Pearson correlation = 0.410, p = 0.05).**

Table 5.2 presents the fluoride concentrations found in saliva and venous plasma and mean difference. Mean (SD) fluoride concentrations in plasma (116.7 (78.3) ng/ml) and whole saliva samples (120.2 (142.3) ng/ml) were much higher than SL-SM saliva (16.5 (16.9) ng/ml). The mean difference between plasma and SL-SM saliva samples was greater (100.2 ng/ml, 5.27 μmol/l) compared to the mean difference between plasma and whole saliva (0.4 ng/ml, 0.02 μmol/l).

As illustrated in Figure 5.5, a negative non-statistically significant correlation between venous plasma and SL-SM saliva was found (Pearson correlation = -0.047, p = 0.836). Figure 5.6 illustrates the correlation between venous plasma and whole saliva. A slight

positive non-statistically significant correlation, was found between venous plasma and whole saliva (Pearson correlation = 0.048, p = 0.832).

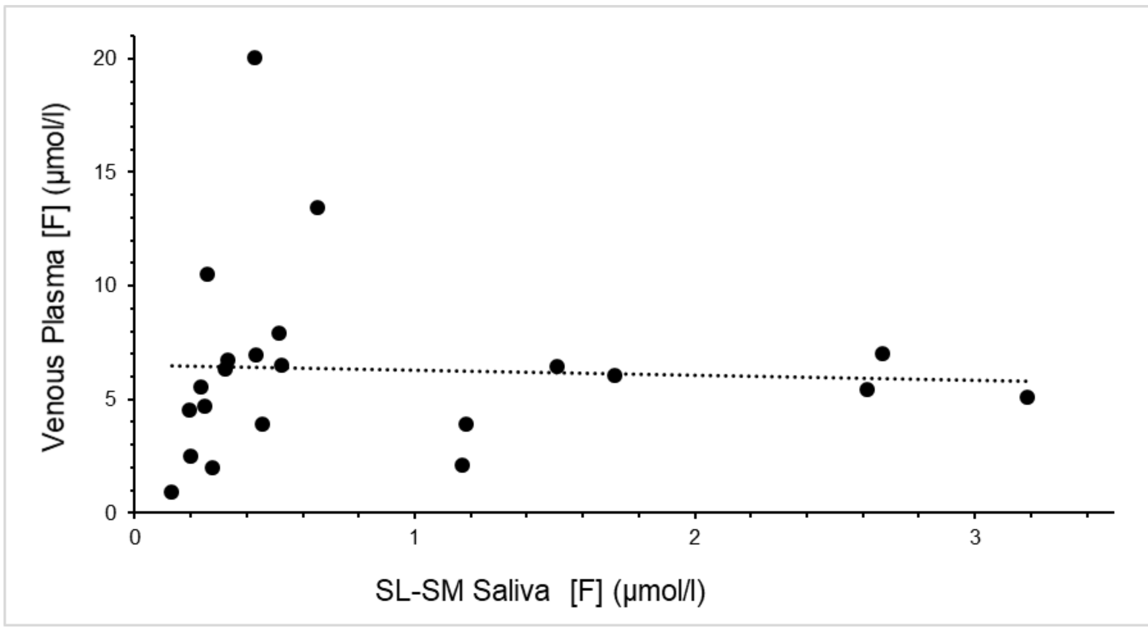


**Figure 5.5. Relation between fluoride concentrations in venous plasma (VP) and SL-SM saliva. VP = 6.491 + (-0.214 x SL-SM). (Pearson correlation = -0.047, p = 0.836).**

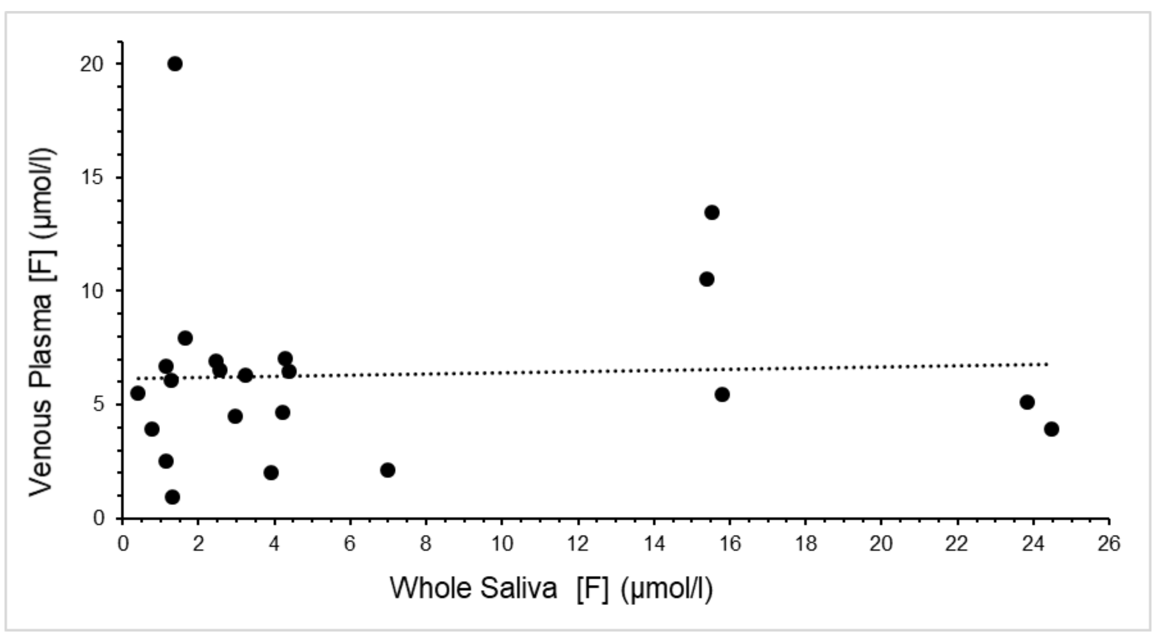


**Figure 5.6. Relation between fluoride concentrations in venous plasma (VP) and whole saliva (WS). VP = 6.136 + (0.027 x WS) (Pearson correlation = 0.048, p = 0.832).**

### 5.9.4. Ratios between saliva and blood fluoride concentrations

The mean ratios between whole saliva and venous plasma, and SL-SM saliva / venous plasma was calculated. A ratio of 1.30 was found between whole saliva and venous plasma. On the other hand, a ratio of 0.18 was found between SL-SM saliva and venous plasma. These ratios were used to calculate predicted plasma fluoride concentrations in section 5.9.5.

### 5.9.5. Predicted plasma fluoride concentration from saliva

Mean actual plasma fluoride concentrations were found to be 6.14 µmol/l. Mean predicted plasma fluoride concentrations were found to be 4.46 µmol/l and 4.81 µmol/l using whole saliva/plasma ratio (1.30) and SL-SM saliva/plasma ratio (0.18), respectively. Table 5.3 presents the mean actual and mean predicted plasma fluoride concentrations.

**Table 5.3. Mean plasma and predicted plasma fluoride concentrations (µmol/l) using whole saliva/plasma ratio (1.30) and SL-SM saliva/plasma ratio (0.18).**

| | Fluoride concentration (µmol/l) | | |
|---|---|---|---|
| | Actual Plasma | Predicted Plasma | |
| | | using whole saliva/plasma ratio* | using SL-SM saliva/plasma ratio** |
| Mean | 6.14 | 4.46 | 4.81 |
| SD | 4.12 | 5.67 | 4.91 |
| Minimum | 0.93 | 0.31 | 0.73 |
| Maximum | 20.04 | 18.82 | 17.62 |

Note:

* Mean Whole Saliva / Venous Plasma Ratio = 1.30
* Mean SL-SM Saliva / Venous Plasma Ratio = 0.18

Figures 5.7 and 5.8 present the correlations between plasma and predicted plasma fluoride concentrations using whole saliva/blood plasma ratio (1.30) and SL-SM saliva / blood plasma ratio (0.18), respectively. There was a statistically non-significant positive correlation between the actual and predicted plasma fluoride concentrations calculated using the whole saliva to venous blood plasma ratio (Pearson correlation = 0.079, p = 0.719). On the other hand, there was a slight negative statistically non-significant correlation between the predicted plasma fluoride concentrations calculated using SL-SM saliva to venous plasma ratio (Pearson correlation = -0.040, p = 0.855).

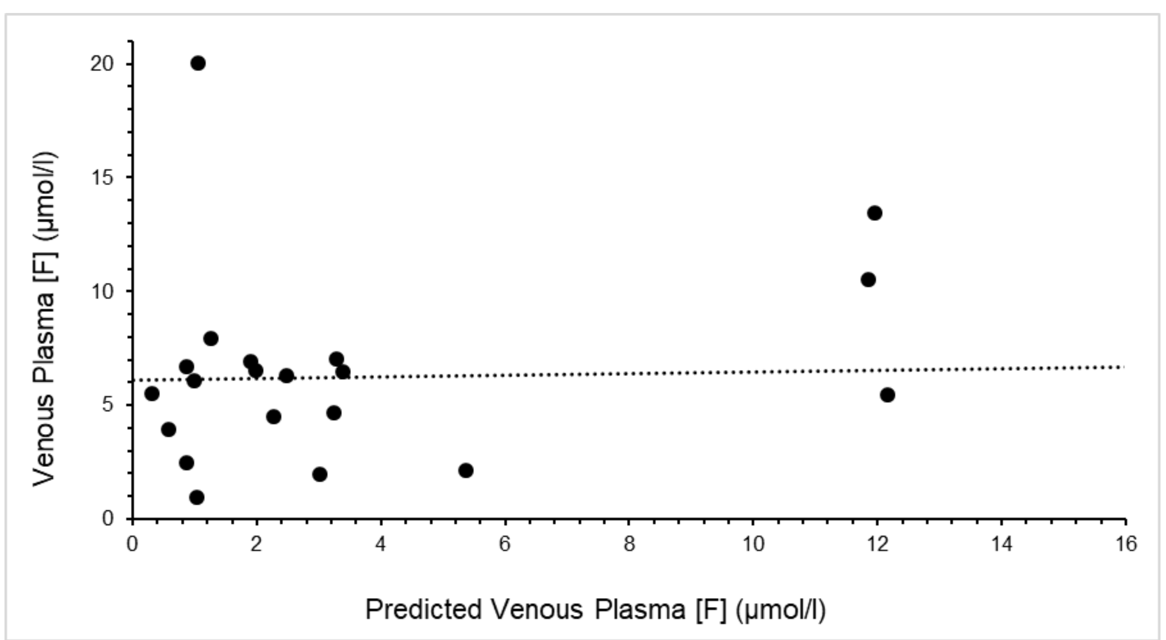


**Figure 5.7. Relation between fluoride concentrations in venous plasma (VP) and predicted venous plasma (PVP), using whole saliva / blood plasma ratio (1.30). VP = 5.8787 + (0.0571 x PVP) (Pearson correlation = 0.079, p = 0.719).**

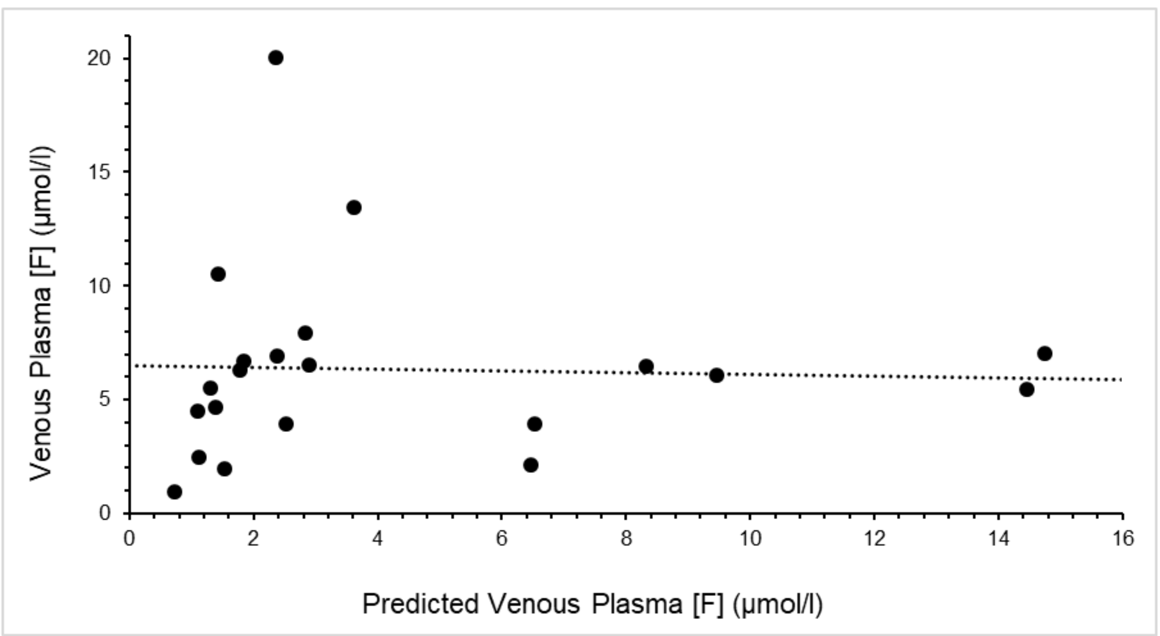


**Figure 5.8. Relation between fluoride concentrations in venous plasma (VP) and predicted venous plasma (PVP), using SL-SM saliva / venous plasma ratio (0.18). VP = 6.3074 + (-0.0399 x PVP). (Pearson correlation = -0.040, p = 0.855).**

## 5.10. Discussion

### 5.10.1. Recruitment

#### 5.10.1.1. Participant

There was an excellent recruitment for this study with a total of 24 participants (9 males and 15 females, aged 18 - 45 years) that took part and completed the data/sample collection. No participants dropped out or were excluded. The good recruitment may have resulted because of the short time commitment and incentive involved. Data and sample collection for the study was completed in under 20 minutes for each participant.

#### 5.10.1.2. Staff

Dental nurses were recruited from the Dental Hospital, School of Dental Sciences at Newcastle University. Both dental nurses were fully trained and experienced in blood collection and had previously collected different forms of saliva's for other research studies. They were able to successfully collect venous blood and saliva samples for this study.

### 5.10.2. Data Collection

Data collection for this study took place in January 2017. Due to the limited availability of the nurses, sample collection was performed in one single session from 8 am till 5 pm. Participants were invited by appointment at 10-minute intervals. Three stations were set up: (1) Blood sampling with dental nurse 1; (2) SL-SM saliva sampling with dental nurse 2; and (3) Whole saliva collection guided by researcher (Maria). Participants moved through the stations and samples were collected in parallel.

The nurses were able to collect blood and SL-SM saliva samples without any challenges or difficulty. During the collection of whole saliva, the majority of participants reported feeling uncomfortable and hesitant to spit or drool into the cup even whilst their privacy was maintained. Participants were given a private area to collect their whole saliva, if they wished to use it.

Following sample collection, participants were casually asked which method out of venous blood, whole saliva and SL-SM saliva collection they preferred. Most participants preferred

venous blood sampling even though it was the most invasive and painful method out of the three. Spitting for the whole saliva collection made few participants uncomfortable to visually see or physically feel their saliva on their lips during collection. Most participants also felt that the SL-SM saliva collection invaded their privacy when dental nurses had to enter their mouths, and this was the least preferred method.

### 5.10.3. Plasma and Saliva

The mean (SD) fluoride concentration of plasma for this study was 6.14 (4.12) µmol/l (n= 24). This is substantially higher than the fluoride concentration of blood plasma found in Study 2 A of 0.71 (0.27) µmol/l (n= 15). Plasma fluoride concentrations in adults in other studies have been found to range between 0.31 and 2.42 µmol/l (Sener *et al.*, (2007); Parkins *et al.* (1974); Cardoso *et al.* (2006); Cardoso *et al.* (2008); Thomas *et al.* (2016); Ekstrand *et al.* (1977)[A]; Ekstrand (1978); Ekstrand *et al.* (1981); Oliveby *et al.* (1989)[1]; Oliveby *et al.* (1989)[2]; Oliveby *et al.* (1989)[3]; Buzalaf *et al.* (2008); Whitford (1996); Whitford *et al.* (2008); Maguire *et al.* (2005)).

The difference in plasma fluoride concentrations in study 2A and 2B could be attributed to several factors. Although, the location of both studies (2A and 2B) and hence the fluoride concentration of the water supply was the same, both studies were performed a year apart. In study 2A, sample collection took place between 9 am till 10 am on a couple of mornings due to the nurse's availability. Whereas, in study 2B sample collection was performed throughout the day from 8 am till 4 pm and was completed within that day, again due to the nurse's availability.

The method of venous blood collection used was the same, however, two different nurses performed the sampling. Fluoride analysis of the samples for both studies was performed by the same researcher (Maria) using the same method i.e. the HMDS acid-diffusion method (Martinez-Mier, *et al.,* 2011). However, both studies and their respective sample analysis was performed during different seasons of the year. Study 2A was performed during the winter, whereas, study 2B was performed during the summer when laboratory conditions were hot and humid. This would often lead to the evaporation of sodium hydroxide (NaOH) drops which were left overnight at room temperature to trap fluoride released from the sample. In such cases, 25 µl 0.1 N acetic acid was added to the sample (as usual) and the final volume of the sample was made to 75 µl using double de-ionised

water ($ddH_2O$) to dissolve the residual NaOH salts. This may have impacted the final fluoride concentration of the sample.

Buzalaf (2011) stated that the concentration of fluoride in plasma samples can be affected by several factors, independent of fluoride dose, such as: site of blood collection; age; acid-base balance; altitude; haematocrit; genetic background; circadian rhythm; and hormones. They explained that the site of blood collection as well as age and haematocrit have the most influence. This is supported by Whitford (1996) who also stated several factors to be responsible for differences in plasma ionic fluoride levels including the site of blood sampling; redistribution of fluoride between blood cells and plasma due to air exposure; associated elevation of blood in blood pH; circadian rhythms.

The age range of study 2B (18 - 45 years) was greater than 2A (19 - 35 years), however the mean age was almost the same i.e. 28.6 years and 29.2 years for 2A and 2B, respectively. The gender distribution was more biased towards males in 2A (n= 15) (10 males and 5 females) and females in 2B (n= 24) (9 males and 15 females), therefore, gender may have also played a role in the varying fluoride concentrations.

Individual diet and toothbrushing habits will have also had an influence on the plasma fluoride concentrations in both studies. However, these may have not differed greatly between individuals from the same geographical area and socio-economic background. Most participants in study 2B were found to have high plasma fluoride concentrations. A study by Whitford *et al.* (2008) found high plasma fluoride concentrations of 7.22 µmol/l in 24 - 32-year olds following the ingestion of a fluoride dose of 2.73 mg. This is equivalent to swallowing approximately 2 grams of toothpaste containing 1450 ppm fluoride. Another study by Buzalaf *et al.* (2008) found high plasma fluoride concentrations of 5.58 µmol/l in 19 - 29-year olds following administration of a fluoride dose of 2 mg (equivalent to swallowing 1.4 grams of toothpaste containing 1450 ppm fluoride). Therefore, the high fluoride concentrations in this study may be attributed to participants having brushed their teeth and swallowed fluoridated toothpaste. It is highly likely that participants brushed their teeth for oral hygiene before arriving for the experimental session as they were also participating in saliva collection during the same session.

Ekstrand (1977)[B] found both plasma and ductal salivary fluoride levels to peak at 30 minutes following a fluoride dose of 3 mg and both remained elevated for up to 8 hours post fluoride ingestion. Whereas, Oliveby *et al.* (1989)[1] and Oliveby *et al.* (1989)[3] found plasma fluoride levels to peak at 40 minutes after a fluoride dose of 1 mg followed by a 10 - 15-minute delay

in peak parotid saliva. Therefore, it is highly likely that the high fluoride levels witnessed in study 2B are reflective of any recent dietary and dentifrice fluoride ingestion.

Three participants had very high plasma fluoride concentrations of 10.51, 13.47 and 20.04 µmol/l. Extremely high plasma fluoride concentrations have previously been found in participants who have been administered high levels of fluoride. A study by Setnikar and Maurer (1990) found maximum plasma fluoride concentrations of 21.67 - 24.51 µmol/l in individuals who were given a single dose of fluoridated products with a fluoride content of 13.2 mg. Although, the participants in the current study were not administered with any source of fluoride, again, diet and dentifrice may have had a large influence on the high plasma fluoride levels obtained.

Although many factors which may be responsible for high fluoride levels witnessed in study 2B have been discussed, the differences in plasma fluoride concentrations between study 2A and study 2B cannot be fully explained.

In the present study, the mean (SD) fluoride concentrations in whole saliva were found to be 6.32 (7.49) µmol/l which was considerably higher than the mean (SD) fluoride concentrations in SL-SM saliva, 0.87 (0.89) µmol/l. The measured mean fluoride concentration of whole saliva in the present study was in line with findings from Fukushima *et al.* (2011) who found whole salivary fluoride concentrations to range from 0.74 µmol/l (equivalent to 14 ng/ml) (age 3 to 7 years) to 15.62 µmol/l (equivalent to 297 ng/ml) (age 14 - 20 years) but were higher than the mean concentrations found by Twetman *et al.* (1998) of 2.2 µmol/l (n= 12) (aged 10 to 13 years) following the consumption of 200 ml of fluoridated milk (1.0 mg F). The high concentrations of fluoride found in whole saliva may have resulted from contamination by diet and fluoride dentifrice which influence the fluoride content in the oral reservoirs (Rølla and Ekstrand, 1996).

The method of collection for whole saliva in this study i.e. passive drool and spit method was simple and straight forward. In terms of practicality, it involved minimal consumables; low cost; no professional personnel / trained staff; non-time consuming and no complicated instructions. From an ethical perspective, it was non-invasive and maintained participant privacy, allowing them to collect their saliva at their own control, in their own time. However, many participants reported feeling uncomfortable whilst collecting their whole saliva.

Mean fluoride concentrations in SL-SM saliva (0.87 µmol/l) found in the present study was similar to concentrations of 0.72 µmol/l found by Twetman *et al.* (1998) (n= 12) (aged 10 to

13 years) following the consumption of 200 ml of fluoridated milk (1.0 mg F). The method of SL-SM saliva collection used by Twetman *et al.* (1998) involved an individual collection device (Block-Brottman device), adapted by Nederfors and Dahlof (1993). However, in the present study, oral syringes were used to collect SL-SM saliva from the base of the mouth under the tongue with the help of Dry Tip pads blocking the parotid duct, which was a new method in the field of fluoride study.

This method may have been prone to slight contamination by whole saliva. Although, there is currently no standard method in literature using oral syringes to collect SL-SM saliva, the use of Dry Tips in blocking the parotid duct should be sufficient to withdraw SL-SM saliva from below the tongue as demonstrated by Nederfors and Dahlof (1993) who used Dry Tips to block the parotid secretions when collecting SL-SM saliva using a modified Block-Brottman device. The main advantages of this method involved low cost and minimal equipment. However, the disadvantages arose from the requirement of trained dental professionals to collect samples. Samples were also prone to contamination due to the lack of control in the method, as mentioned above. The device most widely used in the collection of SL-SM saliva is the Block-Brottman device (Nederfors and Dahlof, 1993; Denny *et al.* (2008); Twetman *et al.* (1998)). The method used by Oliveby *et al.* (1989) using individual saliva collection devices (discussed further) may also be more reliable for the collection of SL-SM saliva. Therefore, further work is required to determine the levels of fluoride detected in SL-SM saliva in the current study along with its relation to plasma.

A non-significant negative correlation was found between fluoride concentrations in whole saliva and SL-SM saliva (Pearson correlation = -0.047, $p = 0.836$) (Figure 5.4) with a mean difference of 5.65 µmol/l. This correlation may have also resulted from the slight contamination of SL-SM saliva with whole saliva due to the method of collection involved i.e. oral syringes, as mentioned above. Apart from the method of cannulation of the SL-SM duct, described by Wolff *et al.* (1997), the other currently available methods are prone to the risk of contamination by other oral fluids.

Participants in this study felt the collection of SL-SM saliva invaded their privacy, however the majority preferred it over whole saliva collection because the sight of their whole saliva during collection made them feel queasy and uncomfortable. Both methods were deemed practical for collection and suitable volumes were gained for fluoride analysis i.e. 2 ml from stimulated SL-SM and 5 ml of unstimulated whole saliva.

The mean (SD) fluoride concentration found in plasma (6.14 (4.12) µmol/l) was similar to the fluoride concentration of whole saliva (6.32 (7.49) µmol/l) but much higher than SL-SM saliva (0.87 (0.89) µmol/l). The lower fluoride concentrations in SL-SM saliva compared to blood plasma may have resulted from the impeded fluoride transport through the acinar cells into the lumen of the salivary gland (Oliveby *et al.*, 1989). The collection of SL-SM saliva in this study is considered as a more reliable indicator of fluoride absorption as oppose to whole saliva which is more prone to contamination of fluoride from other sources.

There was a slight negative correlation between venous blood plasma and SL-SM saliva. Whereas, a slight positive correlation between venous blood plasma and whole saliva was found. A similar study performed by Whitford *et al.* (1999) who investigated the relation between fluoride concentrations in whole saliva, parotid ductal saliva and plasma in 5 to 10-year-old children (n= 17) found no correlation between mean plasma (0.89 µmol/l) and mean whole saliva fluoride levels (7.86 and 5.92 µmol/l, before and after rinsing with deionised water, respectively). However, they did find a strong correlation between plasma and parotid ductal saliva (0.72 µmol/l).

In the present study, the mean difference between plasma and SL-SM saliva samples was greater (5.27 µmol/l) compared to the mean difference between whole saliva and plasma (0.02 µmol/l). Another similar study investigated the relation of fluoride concentrations in SL-SM saliva and plasma in five adults (1 male and 4 females) aged 26 - 38 years (Oliveby *et al.*, 1989). They found unstimulated and stimulated SL-SM saliva fluoride concentrations of 0.44 µmol/l and 0.48 µmol/l to closely relate to corresponding plasma fluoride levels of 0.50 µmol/l and 0.79 µmol/l, respectively. The mean SL-SM saliva fluoride concentration in this study (0.87 µmol/l) was almost twice the levels found by Oliveby *et al.* (1989). The mean (SD) plasma fluoride levels in this study (6.14 (4.12) µmol/l) were also much higher than those reported by Oliveby *et al.* (1989) (0.79 µmol/l). This may be due to the elimination of fluoride ingestion prescribed to participants by Oliveby *et al.* (1989) three days prior to the experiment along with fasting on experimental days. Whereas, participants in this study did not refrain from their usual diet and oral hygiene habits. Therefore, the results will largely be influenced by fluoride exposure from diet and dentifrice, along with fluoride associated with oral reservoirs (Rølla and Ekstrand, 1996).

In the present study, the ratio found between whole saliva / plasma was 1.30. The ratio between SL-SM saliva / plasma on the other hand, was much lower i.e. 0.18. A study by Whitford *et al.* (1999) found mean parotid saliva to plasma ratio of 0.80, which is much

greater than the SL-SM / plasma ratio in this study (0.18). However, the current study investigated SL-SM saliva from adults compared to parotid ductal saliva from children by Whitford *et al.* (1999).

With respect to SL-SM saliva, Oliveby *et al.* (1989) found an unstimulated SL-SM to plasma ratio of 0.55 and stimulated SL-SM to plasma ratio of 0.69. These ratios are again higher compared to the current study. The difference between the ratio found in this study and Oliveby *et al.* (1989) could be attributed to the high plasma fluoride levels witnessed in this study, as discussed previously. The study by Oliveby *et al.* (1989) only had data from five participants, whereas, the current study collected data from 25 participants. However, the method of collection varied, Oliveby *et al.* (1989) used individual saliva collection devices made of silicone impression material. They located the orifices of the SL-SM glands of each individual participant and created the collection devices with a plastic tube inserted close to the orifices of the SL-SM gland ducts.

Predicted mean (SD) plasma fluoride concentrations in this study using the whole saliva / venous blood plasma ratio (1.30) was 4.46 (5.67) µmol/l, compared to 4.81(4.91) µmol/l using the SL-SM saliva / venous blood plasma ratio. Estimated plasma fluoride concentrations using both ratios were very similar. This is supported by findings from Whitford *et al.* (1999) who found almost identical results with a significant linear relationship between plasma and estimated plasma fluoride levels.

Of all three measures used in this study (whole saliva, SL-SM saliva and plasma), plasma is considered as the most preferred method of fluoride absorption levels with great acceptability from participants, for studies on fluoride metabolism in humans. This is due to greater control of the collection method and minimal contamination of the sample, as oppose to both saliva collections. Of both saliva's, SL-SM had greater acceptability and was the most preferred method, as also stated by Buzalaf (2011). Although, it is acknowledged that the collection of SL-SM saliva is technically more difficult (Buzalaf, 2011).

## 5.11. Summary of Findings and Conclusions

The following conclusions were made from the findings of this study:

- Whole saliva and SL-SM saliva can be collected successfully with ease using the drool / spit (without trained staff) and oral syringe method (with trained staff), respectively. Both methods were deemed practical from a collection perspective.
- There was a statistically significant positive correlation (Pearson correlation = 0.410, p = 0.053) between fluoride concentrations in whole saliva and SL-SM saliva.
- Collection of SL-SM saliva is considered as the most preferred method of fluoride absorption from the two saliva measures from these preliminary findings.
- A slight positive non-statistically significant correlation, was found between venous plasma and whole saliva (Pearson correlation = 0.048, p = 0.832).
- There was a slight negative non-statistically significant correlation between venous plasma and SL-SM saliva (Pearson correlation = -0.047, p = 0.836).
- A mean ratio of 1.30 was found between whole saliva and venous plasma.
- A mean ratio of 0.18 was found between SL-SM saliva and venous plasma.
- Overall, plasma is considered as the most preferred measure of fluoride absorption levels for studies on fluoride metabolism in humans.

## 5.12. Overall Conclusion and Recommendations

This study investigated several associations in fluoride concentrations in blood and saliva samples as a measure of fluoride absorption. All collection methods used in study 2A and 2B (capillary blood sampling, venous blood sampling, whole saliva collection and SL-SM saliva collection) were deemed as practical. Sufficient sample for fluoride analysis was gained and sample collection was performed with ease, with the help of trained staff.

Study 2A found venous plasma as the most practical method for collection of blood on studies on fluoride metabolism in humans and this method was then used in study 2B as a comparison with saliva collection (whole saliva and SL-SM saliva). Findings from study 2B found the collection of SL-SM saliva as the most preferred method of saliva collection, however, when compared to plasma, venous plasma was again found to be the most practical and preferred method for studies on fluoride metabolism in humans.

Preliminary findings of this study recommend the use of plasma as a measure of fluoride absorption for studies on fluoride metabolism in humans. However, further work is required to determine fluoride levels and the relations in blood and saliva found.

## 5.13. Future Work

Preliminary findings from study 2A and 2B indicate venous plasma as the most preferred method for studies on fluoride metabolism in humans. However, further work is required to determine the relations found:

- Sample collection should be performed with a larger population size with more controls in place.
- Participants can be prescribed with a fluoride-free regime to eliminate external fluoride exposures when collecting samples.
- Sample collection can be performed upon providing a fluoride dose to test the recovery rate / absorption of fluoride.
- Further work into the collection of SL-SM saliva using other devices can be investigated.
- The collection of parotid ductal saliva can also be investigated.
- Parotid ductal saliva can be compared with SL-SM saliva and plasma.

- Another trial can be conducted to qualitatively and quantitively test participant preference on the collection methods, e.g. questionnaires before and after sample collection, etc.

# Chapter 6. Study 3: The effects of different intensities of continuous exercise on fluoride metabolism in adult humans.

## 6.1. Introduction

Exposures to low levels of fluoride play a major role in the prevention of dental caries. However, many factors can affect the metabolism of fluoride, including physical activity and exercise. Dental caries affects around 60 - 90% of school children worldwide (Petersen and Ogawa, 2016). In England, 12% of three-year olds and 30% of five-year olds are affected by Dental Caries (Public Health England (PHE)[1], 2014; PHE, 2016).

Public health initiatives such as fluoridated milk schemes have been rolled out across schools in order to tackle the problem. An approximate 800,000 school children across five countries worldwide receive fluoridated milk (Banoczy *et al.,* 2009). Over 40,000 school children receive fluoridated milk in the UK alone (Rajpura & Donkin, 2014). However, previous research has indicated that the current UK milk fluoridation scheme does not provide adequate protection for the prevention of dental caries (Ketley and Lennon, 2000). A study by Maguire *et al.* (2013) showed that increasing the school milk fluoride dose to 0.9 mg / 189 ml may still be too low to achieve the World Health Organisation recommended urinary fluoride excretion (UFE) concomitant with optimal fluoride exposure for children <6y (Marthaler, 1999). Fluoridated milk is often provided to school children during their mid-morning break before undergoing physical activity. This may suggest that, the reported low UFE associated with fluoridated milk at doses of 0.5 and 0.9 mg could be related to the effect of physical activity on UFE.

Therefore, this study investigated the effects of exercise on fluoride metabolism in adults. The initial data will be used to design a study protocol investigating the effects of exercise on fluoride metabolism in children. This chapter presents the aims and objectives, methodology, findings and discussion of the main study of this thesis as well as concluding the effects of exercise on fluoride metabolism in adults.

## 6.2. Overall aims and objectives

### 6.2.1. Aims

The main aim of this study was to investigate the effect of an acute session of exercise at different intensities on fluoride metabolic responses in adults.

### 6.2.2. Objectives

The objectives in order to achieve the study aims were as follows:

- To investigate fluoride absorption at different exercise intensities of continuous exercise.
- To investigate urinary fluoride excretion (UFE) at different exercise intensities of continuous exercise over 24-hour period.
- To investigate the relationship between fluoride absorption and excretion at different intensities of continuous exercise.

## 6.3. Materials and Methods

### 6.3.1. Ethical Approval

This project was approved by the Research Governance and Ethics Committee, School of Health and Social Care, Teesside University (Reference number 066/15 - approval attached – Appendix 1). The complete ethics application with supporting documents are also attached (Appendix 2).

### 6.3.2. Study Location

This study took place at Teesside situated in the north east of England. Teesside is primarily a non-fluoridated area, with a water fluoride concentration of <0.3 ppm.

### 6.3.3. Subject

Participants were healthy adults living in non-fluoridated areas from the Teesside area. Both male and female adult participants were recruited. In order to be eligible to participate, the participants had to meet the following inclusion and exclusion criteria:

Inclusion criteria:

- Healthy adult aged between 18 and 35 years;
- No history of acid-base disturbance and not receiving a therapeutic diet;
- Meet the physical activity guidelines from the Chief Medical Office of 150 min of moderate intensity activity per week (Department of Health, Physical Activity, Health Improvement and Protection, 2011) measured by the International Physical Activity Questionnaire (IPAQ);
- Live in any of the non-fluoride postcode areas i.e. TS1 - TS26 (excluding TS17) for at least 3 months prior to and during the study;
- Weigh over 50 Kg.

Exclusion criteria:

- Any symptoms or known presence of cardiovascular, respiratory or metabolic disease (such as high blood pressure, asthma or diabetes);
- Pregnant or planning to become pregnant;
- Musculoskeletal problems i.e. any illness or injury affecting the muscles, tendons, ligaments, nerves, discs, blood vessels, etc.;
- Smoke;
- HIV positive and/or suffer from (carries of) blood borne diseases such as hepatitis C;
- Lactose intolerant;
- Medically advised exercise restrictions;
- Taking any recreational drugs, herbal, homeopathic or any alternative remedies in the last week prior to participation;
- Taking any prescribed medication;
- Home postcode indicated the fluoride concentration in the area is above 0.3 mg/l.

### 6.3.4. Sample size

The target sample size for this exploratory study was 8 - 10 participants based on similar previous studies in humans and animals. A similar pilot study was conducted on the effects of exercise on fluoride metabolism in adult humans by Zohoori *et al.* (2015) with 9 participants (4 male and 5 females: aged 20 - 35 years). Lombarte *et al.* (2013) investigated the effect of physical activity on the toxicity of fluoride on the insulin-glucose system and used 30 rats divided into 3 groups (n= 10 per group) and only one group performed the exercise condition.

### 6.3.5. Recruitment

Participants were recruited from staff and students at Teesside University and their family and friends living in non-fluoridated areas. An invitation email with the participant information sheet attached was circulated by the researcher (Maria Sajjad) via the school of Health and Social Care administrator. An item was also displayed in the Health and Social Care Institute newsletter to advertise the study and invite participants. Those participants interested in taking part contacted the researcher by telephone or email. First, the postcode of the participant was obtained to check for fluoride concentration of the drinking water. If the fluoride concentration was above 0.3 mg/l, the participant was excluded. Secondly, it was ensured that participants met the other inclusion and exclusion criteria. Participants were required to answer the Pre-Activity Readiness Questionnaire (PAR-Q) and International Physical Activity Questionnaire (IPAQ) via email. Participants with no restrictions for exercise according to the PAR-Q and who demonstrated that they meet the UK Chief Medical Office guidelines of 150 minutes of moderate to vigorous physical activity per week according to IPAQ (Bull *et al.,* 2010) were invited to attend. The nature and design of the study was discussed, and participants were given the opportunity to ask any questions or concerns they had regarding the research before obtaining informed consent.

Participants were given an incentive of £100 in Love2Shop gift vouchers at the end of the study for their help in data collection.

### 6.3.6. Pre-experimental Session

Participants were invited to the Laboratory for a pre-experimental session where they had their height and weight measured using a measuring rod with column scale (Seca, 220., Germany). Participants were asked to remove their shoes and all outer clothing such as coats and jackets during weight and height measurements. A $VO_{2\ peak}$ test was performed as part of the background measures. Participants first familiarised themselves with the cycle ergometer. The researcher (Maria Sajjad) verbally informed participants how to use the cycle ergometer followed by allowing them to use the cycle ergometer at a self-selected speed for approximately 5 minutes. A $VO_{2\ peak}$ test was then conducted during the same session to guide the exercise intensities (light, moderate and vigorous) for the experimental sessions.

$VO_{2\ peak}$ refers to the peak oxygen uptake i.e. $VO_{2,}$ during exercise compared to $VO_{2\ max}$ which is defined as the capacity of an individual to consume maximum amount of oxygen from outer atmosphere at a maximal effort in the last thirty seconds of an incremental exercise at sea level (Taylor *et al.,* 1955). The primary criteria for establishing $VO_{2\ max}$ is the attainment of a 'plateau' which is achieved when oxygen uptake, does not increase further following an increase in work load (Howley *et al.,* 1995). However, a $VO_2$ plateau may not always be achieved, depending on the effort exerted by the participant. As research has suggested that the $VO_{2\ peak}$ attained on a maximum-effort incremental test in participants exercising to the limit of tolerance is likely to be a valid index of $VO_{2\ max}$ (Day *et al.*, 2003). Therefore, a $VO_{2\ peak}$ test was chosen to guide the exercise intensities in this study.

**$VO_2$ peak test:** Participants performed a standard cycle ergometer protocol (Evans and White, 2009). Following a 5-minute warm up at 25 Watts, the test started at 25 Watts with the intensity being increased by 25 Watts every 2 minutes until exhaustion. Participants wore a mouthpiece, attached to a Zan online gas analyser (600 USB CPX, nSpire Health Inc., Hertford, UK) which measured the amount of gas exchanged throughout the test. A heart rate monitor RS400 (Polar Electro Oy, Kempele, Finland) was attached to the chest to monitor heart rate (HR). HR was noted every minute and participant rating of perceived exertion (RPE) was noted at the end of every 2-minute stage using a RPE borg scale (Borg, 1998) (Appendix 3).

Participants were encouraged to continue the test for as long as they could until exhaustion, or in some cases the test was discontinued due to the predicted maximum heart rate being achieved ($HR_{max}$ = 220 - age).

The three intensities were determined by an adaptation of the v-slope method (Beaver *et al.,* 1986). Breath by breath data of 1) ventilation (VE) per minute; 2) respiratory exchange ratio (RER) and 3) $CO_2$ output ($VCO_2$) per power (watts), were individually plotted to gain a curve which was divided into two regions to determine the first ventilatory threshold (VT1) and second ventilatory threshold (VT2). VT1 represents the first increase in minute ventilation (VE) that is proportional to the increase in $CO_2$ output ($VCO_2$) (first marked increase along curve) and VT2 represents a high work intensity at which blood lactic accumulation increases accompanied by additional hyperventilation (subsequent increase along curve). This was verified by two researchers (Maria Sajjad and Liane Azevedo).

Exercise intensities were defined for each participant as:

1. Light intensity: one load below VT1
2. Moderate intensity: the load at VT1
3. Vigorous intensity: one load above VT1, which was also one load below VT2

An example illustration of VT1 and VT2 is displayed in figure 6.1.

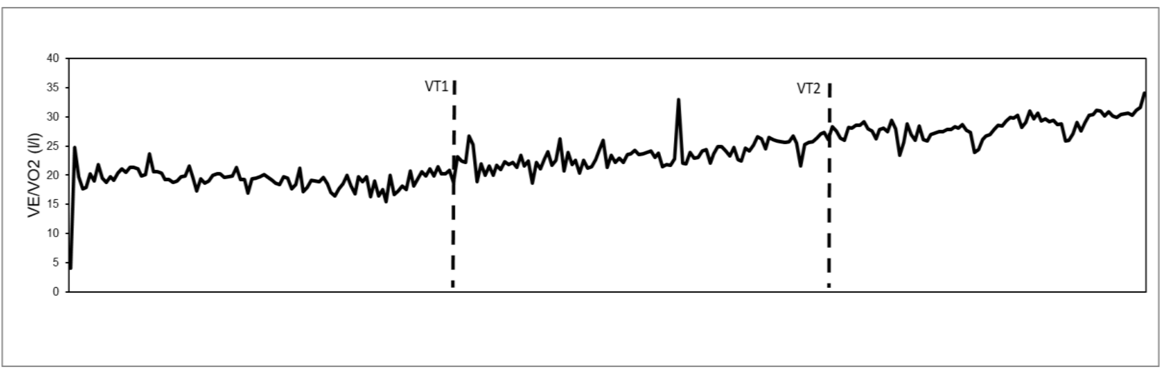


**Figure 6.1. Example illustration of VT1 and VT2 using $VE/VO_2$ (l/l).**

### 6.3.7. Experimental Design

This experiment was designed as a four-treatment repeated measures cross-over study. The study was conducted at the same time of day for each experiment for each subject, to control for circadian rhythms. Each session was separated by a washout period of one week.

Study Exclusion Requirements:

In order to eliminate / minimise fluoride intake / exposure from all other sources during the study, participants were set a fluoride-free regime. They were provided with a fluoride-free toothpaste to use during a one-week wash-out until the period prior to the first experimental session. One week before, as well as during, the whole experimental period, participants were asked to refrain from the following:

- Avoid using any toothpaste, mouthwash containing fluoride or any other significant fluoride products such as fluoride tablets
- Avoid drinking tap water or using tap water in coffee, soups etc. if the Teesside area (TS1 - TS26 and TS29) was left (e.g. to attend a meeting or visit a friend outside these postcodes).
- Avoid drinking tea
- Avoid drinking wine and any beers for at least 48-hours prior to the experiment day
- Only the following alcoholic beverages, although not more than 10 units* per week, were allowed to be consumed for the remainder of the experimental period:
    - Tennents super
    - Heineken
    - McEwans
    - Hooch

* Units

1 pint of lower-strength beer (ABV 3.6%) = 2 units

1 pint of higher-strength beer (ABV 5.2%) = 3 units

1 bottle of beer (330 ml, ABV 5%) = 1.7 units

1 can of beer (440 ml, ABV 4.5%) = 2 units

- All other types of fish apart from saithe and cod fillet. Not more than 500g per week of saithe and cod fillet, were allowed to be consumed. However, these should be avoided for at least 48-hours prior to the experiment day.
- Refrain from any exercise, other than habitual walking, for 48-hours prior to the experiment day.

Experimental Session

Participants were invited to attend the Laboratory at 9:00 am on each experiment day in fasting conditions when the first blood sample was collected. The participants were then provided with a low fluoride breakfast. Breakfast consisting of a cereal bar, banana and fruit juice was provided to all the participants to control the influence of fluoride from diet during the experimental sessions. The breakfast items had been previously tested for fluoride and were found to be low in fluoride (Table 6.1).

**Table 6.1. Fluoride concentration and content of breakfast items provided in the study sessions.**

| **Food** | **Fluoride Concentration (µg/g)** | **Fluoride content per serving unit (µg)** |
|---|---|---|
| Kellogg's – Nutri-grain Golden oat bake | 0.047 | 2.35 |
| Banana | 0.007 | 0.98 |
| Fruit Juice | 0.021 | 2.10 |
| **Total fluoride intake** | **5.43 µg** | |

After breakfast, a heart rate monitor belt (Polar RS400, Finland) was worn on the chest. They were then given one 2.2 mg sodium-fluoride tablet (Endekay Fluotabs 6 years+) with 250 ml low fluoride bottled water (≤0.08 µF/ml). Participants then either rested (control experimental session) or undertook the exercise (experimental sessions) at approximately 9:30am.

Participants warmed up for five minutes at a self-selected speed before initiating the exercise on the cycle ergometers for twenty minutes at one of the following intensities; light, moderate and vigorous intensities (determined at the pre-experimental session). Each experimental session was separated by a "wash-out period" of at least one week. Heart rate was continually monitored during and 10 minutes after exercise.

An overview of the study design is illustrated by Figure 6. 2.

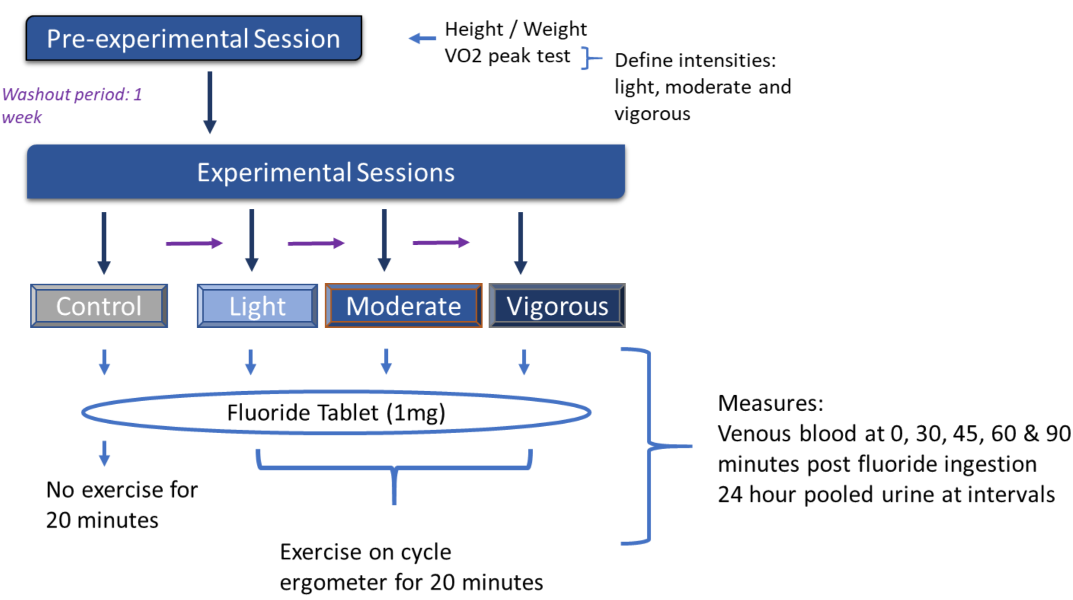


**Figure 6.2 Overview of the study design**

### 6.3.8. Sample collection

**<u>Urine</u>:** Urine was collected by spontaneous voiding during the following time intervals:

1. A pooled urine sample from midnight before the experimental period up until about 9.00 am (9 hours) just before taking the fluoride tablet.
2. A pooled urine sample from 9.00 am to 12.00 pm (3 hours) during the experimental period.
3. A pooled urine sample from 12.00 to 5.00 pm (5 hours) during the experimental period.
4. A pooled sample from 5.00 pm up until midnight (7 hours).

**Blood plasma:** A venous cannula was inserted into the antecubital fossa of either left or right arm and used to collect 5 ml intravenous blood samples to measure fluoride plasma concentrations at the following time points:

1. The first blood sample was obtained prior to fluoride tablet consumption (fasting condition) at time point 0, prior to exercise (for the experimental session).
2. 30 minutes post fluoride ingestion
3. 45 minutes post fluoride ingestion
4. 60 minutes post fluoride ingestion
5. 90 minutes post fluoride ingestion

### 6.3.9. Sample preparation and storage

**Blood:** The blood samples were drawn into 5 ml Vacutainer® spray-coated $K_2$ EDTA blood collection tubes (BD, Oakville) and inverted several times before storage in a refrigerator. The blood samples were centrifuged at 1500 rpm for 10 minutes to separate the plasma. Around 3 ml plasma (supernatant) was separated using a pipette in a fume cupboard and the remaining blood cells / debris were disposed safely into yellow bio-hazardous bins. The 3 ml aliquot was transferred into sterile bijous tubes for fluoride analysis (in triplicate i.e. 3 x 1 ml) and stored in a freezer at -18$^{o}$C until analysis.

**Urine:** Collected urine samples were stored in a refrigerator upon collection till analysis. The volume and pH of each pooled urine samples were measured within 24 - 48 hours of collection and 3 x 10 ml aliquot was taken, and the rest was disposed by flushing down a designated toilet which was cleaned by Virkon solution. The samples were then analysed for fluoride immediately.

### 6.3.10. Sample analysis

The plasma samples were analysed using the HMDS acid-diffusion method – as described in chapter 4 (Martinez-Mier *et al.,* 2011). Whereas, the urine samples were analysed using the direct fluoride ion electrode selective method – as described in chapter 5 (Martinez-Mier *et al.,* 2011). A set of standards was set up using 1ml of 0.01, 0.1, 1, 10 and 100 ppm fluoride in triplicate and ran for analysis using the Fluoride Ion Selective Electrode (Model

Orion 9609BNWP, Thermo Scientific, USA) and meter (Model Orion 720A+, Thermo Electron Corporation, USA).

### 6.3.11. Data handling and analysis

- **Urinary flow rate:** The urinary flow rate was calculated by dividing the urine volume (ml) by duration of collection (hours). The values reported are calculated by dividing urine fluoride excretion over exact duration of urine collection reported by individual participants for each sample, rather than over the duration of each time point.
- **Urinary Fluoride excretion (UFE):** The fluoride excretion of urine samples (µg) was calculated by multiplying the concentration (µg/ml) of the urine sample by the volume (ml) of urine.
- **Rate of Urinary fluoride excretion:** The rate of UFE (µg/hour) was calculated by dividing the fluoride excretion by duration of collection (hours). The values reported were calculated by dividing urine fluoride excretion over exact duration of urine collection reported by individual participants for each sample, rather than over the duration of each time point.
- **$C_{max}$:** $C_{max}$ i.e. maximum fluoride concentration was calculated using the mean maximum baseline corrected plasma fluoride concentration following fluoride dose.
- **$T_{max}$**: approximate $T_{max}$ i.e. lag time of maximum fluoride concentration was estimated using graphs plotting concentration against time (Section 6.4.3).
- **AUC**: Area under the curve was calculated using the following formula: AUC = ∑ 0.5 (plasma fluoride concentration + baseline corrected plasma fluoride concentration) * (time following fluoride dose + baseline corrected time following fluoride dose).
- **Percentage of total daily urinary fluoride excretion:** The percentage of total daily UFE (%) was calculated by (dividing mean rate of UFE (µg/hour) for a given time point, by the total rate of UFE (µg/hour) post fluoride ingestion) * 100.

#### 6.3.11.1. Statistical Analysis

Raw data was analysed using Microsoft Excel spreadsheets. Microsoft Excel was also used to construct graphs to descriptively illustrate the following trends for all experimental sessions:

- Mean plasma fluoride concentrations across 90 minutes post fluoride ingestion
- Mean urinary pH levels throughout the day
- Baseline corrected rate of UFE over 24-hour period

Descriptive statistics was performed using statistical software, IBM SPSS Statistics (version 24). A linear mixed model analysis was conducted to gain estimated marginal means (Mean and standard deviations) and pairwise comparisons. The pairwise comparisons (95% CI, upper and lower limits, along with P values) were used to construct figures to compare the analysis of variation for $T_{max}$ (mins) post fluoride ingestion; $C_{max}$ (ng/ml) post fluoride ingestion; Area under the curve (AUC) (µg/min/ml-1) for 0 - 3 hours post fluoride ingestion; and rate of UFE (µg/hour) for 0 - 3, 3 - 5 and 8 - 24 hours post fluoride ingestion, across the following experimental sessions:

- Control vs Light
- Control vs Moderate
- Control vs Vigorous
- Light vs Moderate
- Light vs Vigorous
- Vigorous vs Moderate

## 6.4. Results

### 6.4.1. Recruitment

Recruitment for this study was undertaken between May and December 2016. Recruitment material was circulated amongst staff and students across the five schools/departments of Teesside University. Approximately 2,396 staff and 18,576 students (full time and part time) were contacted and invited to take part. Invitations were also open to family and friends of staff and students at Teesside University.

Of all those invited, 16 people (51.6%) showed initial interest. Following the recruitment process, only 13 (41.9%) signed consent and started the study, of which 3 (23%) dropped out due to time constraints and other commitments and a further 2 (15%) were excluded due to health and safety / medical reasons. In the end, only 8 participants (61.5%) completed the study. Table 6.2 presents age and anthropometric data relating to the 8 participants who completed the study. Table 6.3 presents information relating the exercise intensities resulting from the $VO_{2\ max}$ test for all the participants during recruitment, along with the maximum HR and RPE reported during the $VO_{2\ max}$ test.

**Table 6.2. Participant age and anthropometric data (n= 8) (male: female = 4:4).**

| Subject ID | Gender | Age (years) | Height (cm) | Weight (kg) | BMI (kg/m2) |
|---|---|---|---|---|---|
| 1 | Male | 35 | 172.0 | 69.0 | 23.3 |
| 2 | Male | 22 | 186.0 | 84.7 | 24.4 |
| 4 | Male | 21 | 175.5 | 81.9 | 26.5 |
| 5 | Female | 35 | 161.0 | 63.8 | 24.6 |
| 6 | Male | 22 | 171.5 | 61.1 | 20.7 |
| 9 | Female | 18 | 164.5 | 66.9 | 24.7 |
| 10 | Female | 18 | 166.0 | 65.7 | 23.8 |
| 11 | Female | 24 | 170.5 | 60.5 | 20.8 |

| **Mean** | **-** | **24** | **170.9** | **69.2** | **23.6** |
|---|---|---|---|---|---|

**Table 6.3. Exercise Intensities following $VO_{2\ max}$ test during recruitment. Maximum heart rate (HR) measured and RPE (Rate of Perceived Exertion) reported by participants at end of the $VO_{2\ max}$ test are also reported.**

| **Subject ID** | **Exercise Load (Watts)** | | | **Max HR** | **RPE** |
|---|---|---|---|---|---|
| | **Light** (one load below VT1) | **Moderate** (at VT1) | **Vigorous** (one load below VT2) | | |
| 1 | 25 | 50 | 75 | 117 | 9 |
| 2 | 50 | 100 | 150 | 183 | 7 |
| 4 | 125 | 150 | 175 | 174 | 9 |
| 5 | 25 | 50 | 75 | 163 | 5 |
| 6 | 75 | 125 | 150 | 169 | 6 |
| 9 | 50 | 75 | 100 | 183 | 4 |
| 10 | 50 | 75 | 100 | 193 | 4 |
| 11 | 125 | 150 | 175 | 165 | 6 |
| **Mean** | **65.6** | **96.9** | **125** | **168.4** | **6.3** |

### 6.4.2. Plasma

Tables 6.4 A to 6.4 D present the individual plasma fluoride concentrations for all experimental sessions: 6.4A – Control, 6.4B – Light, 6.4C – Moderate and 6.4D – Vigorous. The individual plasma fluoride concentrations, corrected for baseline, for all experimental sessions are presented in table 6.5: 6.5A – Control, 6.5B – Light, 6.5C – Moderate and 6.5D – Vigorous.

**Table 6.4. Plasma fluoride concentrations in ng/ml and μmol/l (in brackets) for all subjects (n= 8), individually, at pre- and post-fluoride ingestion.**

A. Plasma fluoride concentrations [ng/ml (μmol/l)] for **control** experimental sessions.

| Participant | Plasma fluoride Concentration [ng/ml (μmol/l)] at | | | | |
|---|---|---|---|---|---|
| | Pre-fluoride ingestion | Post fluoride ingestion (minutes) | | | |
| | 0 | 30 | 45 | 60 | 90 |
| 1 | 2.6 (0.14) | 2.1 (0.11) | 7.9 (0.42) | 10.9 (0.58) | 7.9 (0.42) |
| 2 | 1.3 (0.07) | 2.0 (0.11) | * | * | * |
| 4 | 1.3 (0.07) | 19.1 (1.01) | 11.7 (0.62) | 7.1 (0.38) | 11.4 (0.60) |
| 5 | 13.9 (0.73) | 32.4 (1.71) | 53.0 (2.80) | 23.0(1.21) | 21.4 (1.13) |
| 6 | 1.2 (0.06) | 11.2 (0.59) | 28.5 (1.50) | 9.7 (0.51) | 13.9 (0.73) |
| 9 | 16.2 (0.85) | 30.3 (1.59) | 33.0 (1.74) | 60.1 (3.16) | * |
| 10 | 3.1 (0.16) | 42.6 (2.24) | 46.4 (2.44) | 55.0 (2.90) | 38.2 (2.01) |
| 11 | 8.0 (0.42) | 23.3 (1.22) | 26.4 (1.39) | 69.4 (3.65) | 6.6 (0.35) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

B. Plasma fluoride concentrations [ng/ml (μmol/l)] for **light** experimental sessions.

| Participant | Plasma fluoride Concentration [ng/ml (μmol/l)] at | | | | |
|---|---|---|---|---|---|
| | Pre-fluoride ingestion | Post fluoride ingestion (minutes) | | | |
| | 0 | 30 | 45 | 60 | 90 |
| 1 | 67.9 (3.57) | 171.9 (9.05) | 151.6 (7.98) | 91.2 (4.80) | 120.7 (6.35) |
| 2 | 44.3 (2.33) | 103.4 (5.44) | 165.0 (8.69) | 73.3 (3.86) | 59.1 (3.11) |
| 4 | 57.9 (3.05) | 116.8 (6.15) | 250.1 (13.17) | 339.0 (17.84) | 295.8 (15.57) |
| 5 | * | 134.7 (7.09) | 90.8 (4.78) | 36.4 (1.92) | 18.2 (0.96) |
| 6 | 32.4 (1.70) | 111.0 (5.84) | 106.1 (5.59) | 159.0 (8.37) | 120.3 (6.33) |
| 9 | 100.1 (5.27) | 262.0 (13.79) | 236.0 (12.42) | 184.1 (9.69) | 151.0 (7.95) |
| 10 | * | * | * | * | * |
| 11 | 14.1 (0.74) | 156.5 (8.23) | 35.9 (1.89) | 59.0 (3.10) | 5.1 (0.27) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

C. Plasma fluoride concentrations [ng/ml (μmol/l)] for **moderate** experimental sessions.

| Participant | Plasma fluoride Concentration [ng/ml (μmol/l)] at | | | | |
|---|---|---|---|---|---|
| | Pre-fluoride ingestion | Post fluoride ingestion (minutes) | | | |
| | 0 | 30 | 45 | 60 | 90 |
| 1 | 32.1 (1.69) | 222.8 (11.73) | 102.7 (5.41) | 160.9 (8.47) | 250.8 (13.20) |
| 2 | 84.5 (4.45) | 159.6 (8.40) | 268.7 (14.14) | 177.4 (9.34) | 84.0 (4.42) |
| 4 | 30.4 (1.60) | 208.7 (10.99) | 248.0 (13.05) | 280.4 (14.76) | 231.1 (12.16) |
| 5 | 28.6 (1.51) | 517.6 (27.24) | 456.2 (24.01) | 332.7 (17.51) | 261.5 (13.76) |
| 6 | 32.0 (1.69) | 115.6 (6.09) | 165.6 (8.72) | 289.6 (15.24) | 157.9 (8.31) |
| 9 | 6.3 (0.33) | 208.3 (10.96) | 269.1 (14.16) | 148.3 (7.81) | 320.5 (16.87) |
| 10 | * | * | * | * | * |
| 11 | 44.4 (2.34) | 256.6 (13.50) | 331.3 (17.44) | 281.3 (14.81) | 130.9 (6.89) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

D. Plasma fluoride concentrations [ng/ml (µmol/l)] for **vigorous** experimental sessions.

| Participant | Plasma fluoride Concentration [ng/ml (µmol/l)] at | | | | |
|---|---|---|---|---|---|
| | Pre-fluoride ingestion | Post fluoride ingestion (minutes) | | | |
| | 0 | 30 | 45 | 60 | 90 |
| 1 | 42.1 (2.21) | 64.4 (3.39) | 176.5 (9.29) | 92.0 (4.84) | 143.8 (7.57) |
| 2 | 53.9 (2.83) | 64.2 (3.38) | 97.3 (5.12) | 104.5 (5.50) | 87.2 (4.59) |
| 4 | 53.7 (2.83) | 76.4 (4.02) | 133.8 (7.04) | 162.8 (8.57) | 118.6 (6.24) |
| 5 | 37.6 (1.98) | 133.3 (7.02) | 241.4 (12.70) | 129.8 (6.83) | 151.0 (7.95) |
| 6 | 22.4 (1.18) | 70.2 (3.70) | 59.4 (3.13) | 117.3 (6.17) | 84.3 (4.44) |
| 9 | * | * | * | * | * |
| 10 | 58.9 (3.10) | 91.8 (4.83) | 148.8 (7.83) | 149.8 (7.88) | 39.6 (2.09) |
| 11 | 30.3 (1.60) | 156.3 (8.23) | 101.1 (5.32) | 136.0 (7.16) | 154.9 (8.15) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

**Table 6.5. Plasma fluoride concentrations in ng/ml and µmol/l (in brackets), corrected for baseline for all subjects (n=8), individually, at post-fluoride ingestion.**

A. Plasma fluoride concentrations [ng/ml (µmol/l)], corrected for baseline for **control** experimental session.

| Participant | Plasma fluoride Concentration [ng/ml (µmol/l)] at Post fluoride ingestion (minutes) | | | |
|---|---|---|---|---|
| | 30 | 45 | 60 | 90 |
| 1 | -0.5 (-0.03) | 5.3 (0.28) | 8.3 (0.44) | 5.2 (0.28) |
| 2 | 0.7 (0.04) | * | * | * |
| 4 | 17.8 (0.94) | 10.4 (0.55) | 5.8 (0.31) | 10.1 (0.53) |
| 5 | 18.5 (0.98) | 39.1 (2.06) | 9.1 (0.48) | 7.5 (0.40) |
| 6 | 10.0 (0.53) | 27.3 (1.44) | 8.5 (0.45) | 12.7 (0.67) |
| 9 | 14.1 (0.74) | 16.9 (0.89) | 43.9 (2.31) | * |
| 10 | 39.6 (2.08) | 43.3 (2.28) | 51.9 (2.73) | 35.2 (1.85) |
| 11 | 15.3 (0.81) | 18.4 (0.97) | 61.4 (3.23) | -16.7 (-0.88) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

B. Plasma fluoride concentrations [ng/ml (μmol/l)], corrected for baseline for **light** experimental session.

| Participant | Plasma fluoride Concentration [ng/ml (μmol/l)] at Post fluoride ingestion (minutes) | | | |
|---|---|---|---|---|
| | **30** | **45** | **60** | **90** |
| 1 | 104.0 (5.47) | 83.7 (4.41) | 23.3 (1.23) | 52.8 (2.78) |
| 2 | 59.2 (3.11) | 120.8 (6.36) | 29.0 (1.53) | 14.9 (0.78) |
| 4 | 58.9 (3.10) | 192.2 (10.12) | 281.1 (14.79) | 237.9 (12.52) |
| 5 | 134.7 (7.09) | 90.8 (4.78) | 36.4 (1.92) | 18.2 (0.96) |
| 6 | 78.6 (4.14) | 73.8 (3.88) | 126.7 (6.67) | 88.0 (4.63) |
| 9 | 161.9 (8.52) | 135.9 (7.16) | 84.1 (4.42) | 50.9 (2.68) |
| 10 | * | * | * | * |
| 11 | 142.4 (7.49) | 21.8 (1.15) | 44.9 (2.36) | -9.0 (-0.47) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

C. Plasma fluoride concentrations [ng/ml (μmol/l)], corrected for baseline for **moderate** experimental session.

| Participant | Plasma fluoride Concentration [ng/ml (μmol/l)] at Post fluoride ingestion (minutes) | | | |
|---|---|---|---|---|
| | 30 | 45 | 60 | 90 |
| 1 | 190.7 (10.04) | 70.6 (3.72) | 128.8 (6.78) | 218.7 (11.51) |
| 2 | 75.1 (3.95) | 184.2 (9.70) | 92.9 (4.89) | -0.4 (-0.02) |
| 4 | 178.3 (9.39) | 217.6 (11.45) | 249.9 (13.16) | 200.7 (10.56) |
| 5 | 489.0 (25.74) | 427.5 (22.50) | 304.1 (16.01) | 232.8 (12.25) |
| 6 | 83.6 (4.40) | 133.6 (7.03) | 257.6 (13.56) | 125.8 (6.62) |
| 9 | 202.0 (10.63) | 262.8 (13.83) | 142.1 (7.48) | 314.2 (16.54) |
| 10 | * | * | * | * |
| 11 | 212.2 (11.17) | 286.9 (15.10) | 237.0 (12.47) | 86.50 (4.55) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

D. Plasma fluoride concentrations [ng/ml (µmol/l)], corrected for baseline for **vigorous** experimental session.

| Participant | Plasma fluoride Concentration [ng/ml (µmol/l)] at Post fluoride ingestion (minutes) | | | |
|---|---|---|---|---|
| | **30** | **45** | **60** | **90** |
| 1 | 22.3 (1.18) | 134.5 (7.08) | 50.0 (2.63) | 101.8 (5.36) |
| 2 | 10.4 (0.55) | 43.4 (2.29) | 50.6 (2.67) | 33.3 (1.76) |
| 4 | 22.7 (1.19) | 80.1 (4.22) | 109.1 (5.74) | 64.9 (3.42) |
| 5 | 95.8 (5.04) | 203.8 (10.73) | 92.3 (4.86) | 113.5 (5.97) |
| 6 | 47.8 (2.52) | 37.0 (10.73) | 94.9 (4.86) | 62.0 (5.97) |
| 9 | * | * | * | * |
| 10 | 33.0 (1.73) | 89.9 (4.73) | 90.8 (4.78) | -19.3 (-1.02) |
| 11 | 126.0 (6.63) | 70.8 (3.73) | 105.7 (5.56) | 124.6 (6.56) |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

Table 6.6 presents the mean baseline plasma fluoride concentration for control, light, moderate and vigorous experimental sessions. The mean baseline values for light, moderate and vigorous experimental sessions were all fairly similar. However, the mean (SD) baseline plasma fluoride concentration for the control experimental session (5.9 (6.1) ng/ml) was lower compared to the light (45.2 (33.8) ng/ml), moderate (36.9 (23.9) ng/ml) and vigorous (42.7 (13.6) ng/ml) experimental sessions.

**Table 6.6. Mean (SD) plasma fluoride (F) concentrations [ng/ml (μmol/l)*] for all subjects (n= 8) by experimental session.**

| Collection time | Mean plasma fluoride concentration (ng/ml) (SD) *[μmol/l (SD)]* | | | |
|---|---|---|---|---|
| | Control | Light | Moderate | Vigorous |
| Baseline (pre-F ingestion) | 5.9 (6.1) *[0.31 (0.32)]* | 45.2 (33.8) *[2.38 (1.79)]* | 36.9 (23.9) *[1.94 (1.26)]* | 42.7 (13.6) *[2.25 (0.71)]* |
| 30 minutes post-F ingestion | 20.4 (14.7) *[1.07 (0.77)]* | 150.9 (54.9) *[7.94 (2.89)]* | 241.3 (130.1) *[12.70 (6.85)]* | 93.8 (36.7) *[4.94 (1.93)]* |
| 45 minutes post-F ingestion | 29.6 (16.6) *[1.56 (0.87 )]* | 147.9 (77.5) *[7.79 (4.08)]* | 263.1 (113.6) *[13.85 (5.98)]* | 136.9 (59.8) *[7.21 (3.15)]* |
| 60 minutes post-F ingestion | 33.6 (26.9) *[1.77 (1.42 )]* | 134.6 (104.7) *[7.08 (5.51)]* | 238.7 (74.1) *[12.56 (3.90)]* | 127.5 (24.9) *[6.71 (1.31)]* |
| 90 minutes post-F ingestion | 16.6 (12.5) *[0.87 (0.62)]* | 110.0 (98.7) *[5.79 (5.19)]* | 205.2 (83.3) *[10.80 (4.39)]* | 111.4 (42.9) *[5.86 (2.26)]* |

**Note:*** Figures in italic present the result in μmol/l (SD)

Table 6.7 presents mean plasma fluoride concentrations, corrected for baseline for all experimental sessions. The highest mean plasma fluoride concentrations were found for the moderate exercise experimental session with the mean (SD) peak of 226.2 (115.6) ng/ml, [11.90 (6.08) μmol/l] at 45 minutes post fluoride ingestion.

**Table 6.7. Mean (SD) plasma fluoride (F) concentrations in ng/ml and μmol/l* [in brackets], corrected for baseline, for all subjects (n= 8) by experimental session.**

| Collection time | Mean Fluoride concentration (ng/ml) (SD) *[μmol/l (SD)]* | | | |
|---|---|---|---|---|
| | **Control** | **Light** | **Moderate** | **Vigorous** |
| 30 minutes post-F ingestion | 14.4 (12.5) *[0.76 (0.66)]* | 105.7 (41.7) *[5.56 (2.20)]* | 204.4 (137.4) *[10.76 (7.23)]* | 51.1 (43.3) *[2.69 (2.28)]* |
| 45 minutes post-F ingestion | 23.6 (14.3) *[1.24 (0.75)]* | 102.7 (58.2) *[5.41 (3.06)]* | 226.2 (115.6) *[11.90 (6.08)]* | 94.2 (58.1) *[4.96 (3.06)]* |
| 60 minutes post-F ingestion | 27.7 (24.3) *[1.46 (1.28)]* | 89.3 (96.6) *[4.70 (5.09)]* | 201.8 (79.4) *[10.62 (4.18)]* | 84.8 (24.5) *[4.46 (1.29)]* |
| 90 minutes post-F ingestion | 10.6 (15.5) *[0.56 (0.82)]* | 64.8 (87.2) *[3.41 (4.59)]* | 168.3 (104.9) *[3.61 (5.52)]* | 68.7 (50.5) *[3.61 (2.66)]* |

**Note:** * Figures in italic present the result in μmol/l

The mean plasma fluoride concentrations across the 90 minutes post fluoride ingestion for all experimental sessions are also illustrated by figure 6.3. All experimental sessions follow a similar trend, peaking between 30 to 60 minutes post fluoride ingestion. Slightly later peaks are produced by the control and vigorous experimental sessions compared to light and moderate experimental sessions. The highest concentrations are displayed by the moderate experimental session.

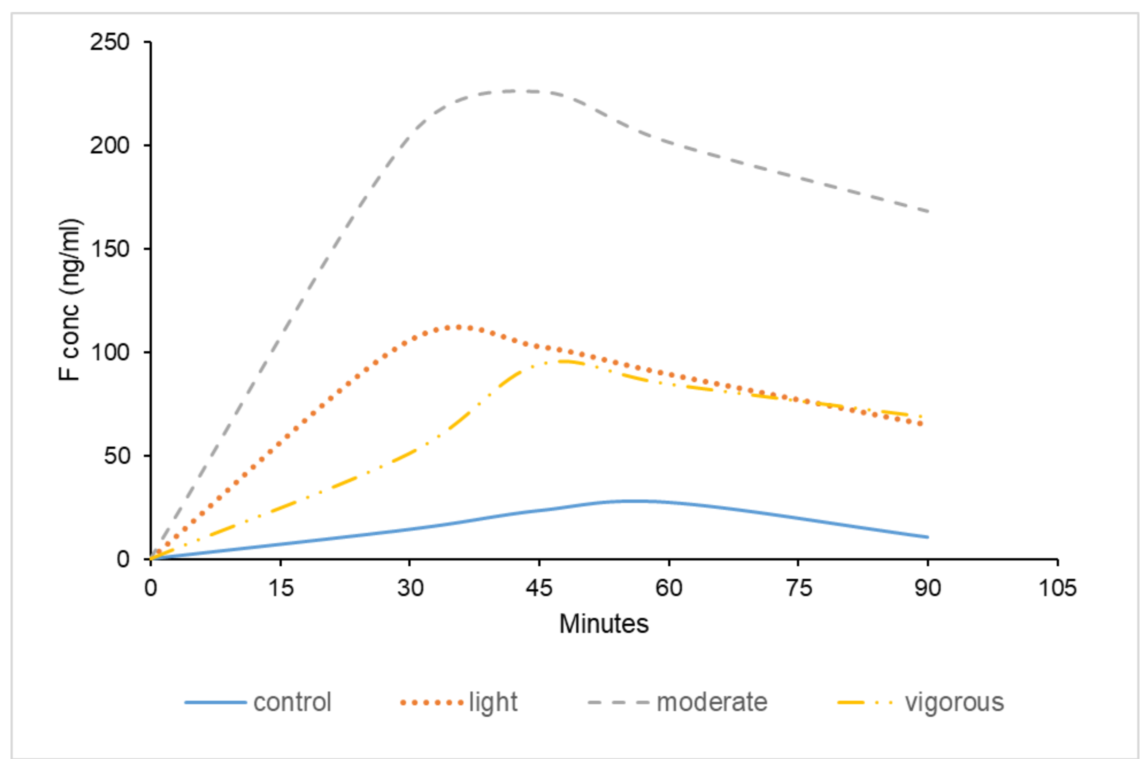


**Figure 6.3. Plasma Fluoride (F) Concentrations (ng/ml) across the 90 minutes post fluoride ingestion for all experimental sessions. Key: Control – Blue; Light – Orange; Moderate – Grey and Vigorous – Yellow.**

Peak concentrations ($C_{max}$) and time points of peak concentration ($T_{max}$) for each experimental session are presented in Table 6.8. The following trends were found. The highest mean $C_{max}$ as mentioned arose from the moderate exercise experimental session of 226.2 ng/ml. This was followed by the light exercise experimental session mean $C_{max}$ of 102.7 ng/ml. Vigorous exercise and the control experimental session had much lower mean $C_{max}$ values of 94.2 ng/ml and 23.6 ng/ml, respectively.

The mean $T_{max}$, on the other hand, increased as the intensity of exercise increased resulting in a slightly delayed peak for the vigorous experimental session (50 minutes) compared to all other experimental sessions which peaked only around a minute apart from one another. The mean $T_{max}$ for the control experimental session was 44 minutes, light experimental session was 45 minutes and moderate experimental session with the highest $C_{max}$ was also at 45 minutes.

**Table 6.8. Mean (95% Confidence Interval (CI)) pharmacokinetics of fluoride (plasma Tmax, Cmax and AUC (0-90 minutes)) following ingestion of fluoride tablet (2.2 mg) by experimental session.**

| Experimental session | $T_{max}$ (minutes) | $C_{max}$ (ng/ml) | AUC 0-90 minutes (ng.min.ml$^{-1}$) |
|---|---|---|---|
| Control | 44 (38, 50) | 23.6 (5.195, 50.205) | 1584.0 (-1430.4, 4598.4) |
| Light | 45 (38, 51) | 102.7 (67.098, 144.245) | 8328.6 (5450.5, 11206.7) |
| Moderate | 45 (40, 51) | 226.2 (119.275, 333.090) | 14472.5 (11746.6, 17198.4) |
| Vigorous | 50 (45, 56) | 94.2 (40.487, 147.939) | 6025.2 (3376.8, 8673.5) |

Figure 6.4 illustrates a comparison of the analysis of variation for $T_{max}$ (minutes) post fluoride ingestion for all the experimental sessions. No statistically significant differences in $T_{max}$ were found between all sessions ($p > 0.05$).

Figure 6.5, on the other hand illustrates a comparison of the analysis of variation for $C_{max}$ post fluoride ingestion for all the experimental sessions. Statistically significant differences in $C_{max}$ were found between control and moderate ($p = 0.001$) and light and moderate ($p = 0.010$). No other statistically significant differences in $C_{max}$ for the remaining sessions were found.

The analysis of variation for AUC for 0 - 90 minutes post fluoride ingestion for all the experimental sessions is illustrated by Figure 6.6. Statistically significant differences in AUC were found between control and all three exercise sessions (light, moderate and vigorous) ($p < 0.05$); whereas there was no statistically significant difference in AUC between light and vigorous exercise ($p = 0.222$).

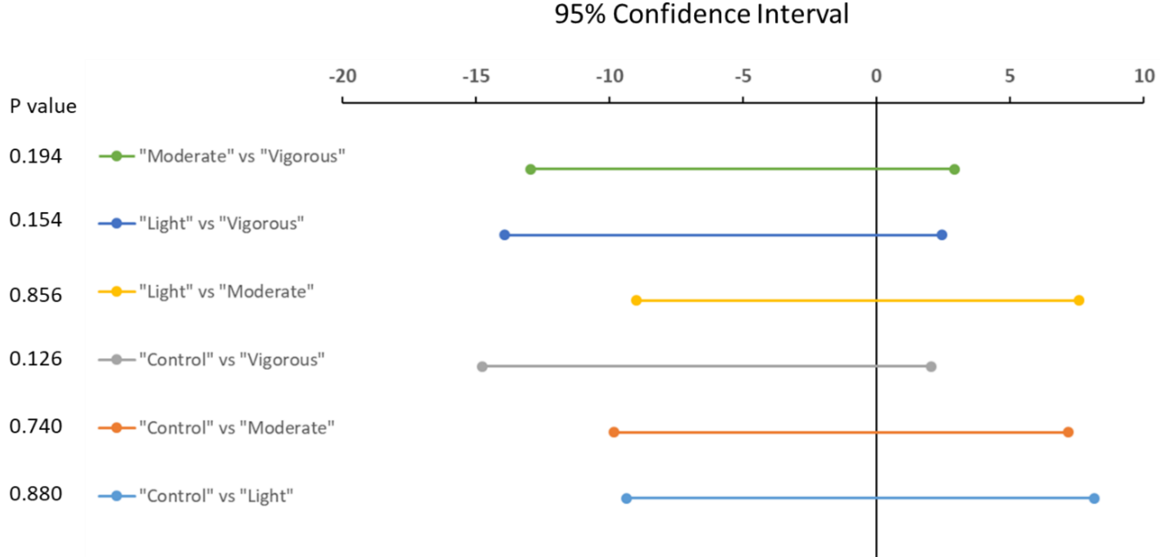


**Figure 6.4. Analysis of variation across all experimental sessions for $T_{max}$ (mins) post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. 95% confidence interval of differences, lower and upper limit values have been reported.**

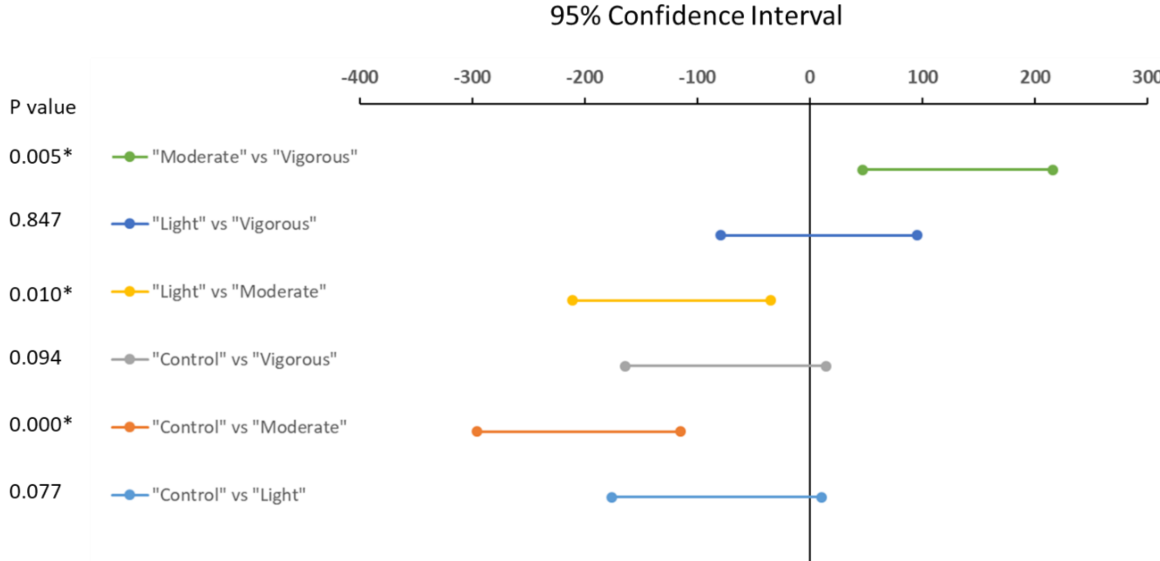


**Figure 6.5. Analysis of variation across all experimental sessions for $C_{max}$ (ng/ml) post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. Statistically significant values are marked with an asterisk (*). 95% confidence interval of differences, lower and upper limit values have been reported.**

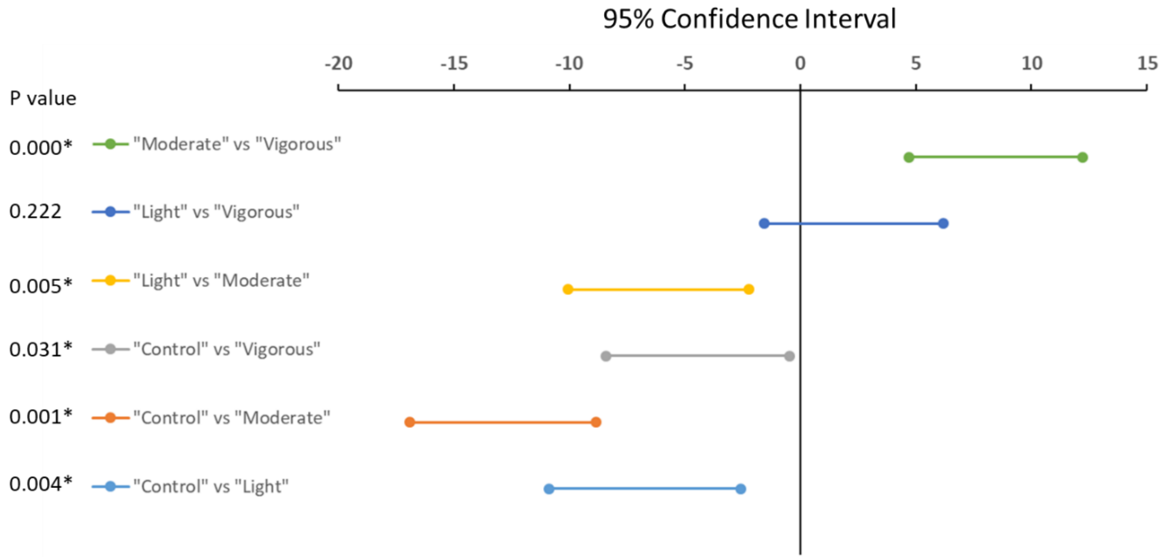


**Figure 6.6. Analysis of variation across all experimental sessions for Area under the curve (AUC) (μg/min/ml$^{-1}$) for 0 – 90 minutes post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. Statistically significant values are marked with an asterisk (*). 95% confidence interval of differences, lower and upper limit values have been reported.**

### 6.4.3. Urine

#### 6.4.3.1. Urinary flow rate

Table 6.9 presents mean (SD) urinary flow rate as well as that when corrected for body weight, for all experimental sessions. The mean urinary flow rate for baseline varied for all four experimental sessions, ranging from 30.1 - 55.6 ml/hour. Mean urinary flow rate for the control session (75.6 (40.9) ml/hour) peaked between 9am - 12pm, whereas for light (91.7 (31.3) ml/hour), moderate (63.4 (29.9) ml/hour) and vigorous (80.5 (54.1) ml/hour) exercise it peaked slightly later, between 12pm - 5pm.

**Table 6.9. Mean (SD) Urinary flow rate (ml/hour), unadjusted and corrected for body weight, for all experimental sessions.**

| Time point (Mean duration of collection: hour) | Control | Light | Moderate | Vigorous |
|---|---|---|---|---|
| **Urinary flow rate (SD) (ml/hour) [N]** | | | | |
| 00:00 - 9:00 Baseline (9 hours) | 38.0 (25.9) [8] | 45.3 (28.0) [6] | 30.1 (28.7) [7] | 55.6 (22.2) [7] |
| 9:00 - 12:00 (3 hours) | 75.6 (40.9) [6] | 54.4 (30.3) [6] | 40.4 (36.4) [6] | 45.5 (28.1) [7] |
| 12:00 - 17:00 (5 hours) | 55.9 (36.6) [8] | 91.7 (31.3) [6] | 63.4 (29.9) [6] | 80.5 (54.1) [6] |
| 17:00 - 24:00 (7 hours) | 44.1 (26.3) [8] | 39.9 (29.0) [7] | 47.0 (26.2) [7] | 47.3 (34.2) [7] |
| **Body weight corrected, Urinary flow rate (SD) (ml/ kg bw/ hour)** | | | | |
| 00:00 - 9:00 Baseline (9 hours) | 0.5 (0.3) | 0.5 (0.5) | 0.4 (0.5) | 0.7 (0.5) |
| 9:00 - 12:00 (3 hours) | 0.8 (0.7) | 0.6 (0.5) | 0.5 (0.6) | 0.6 (0.4) |
| 12:00 - 17:00 (5 hours) | 0.8 (0.6) | 1.0 (0.8) | 0.7 (0.6) | 0.9 (0.9) |
| 17:00 - 24:00 (7 hours) | 0.6 (0.4) | 0.5 (0.4) | 0.6 (0.4) | 0.6 (0.6) |

#### 6.4.3.2. pH of urine

Figure 6.7 illustrates the mean pH levels of urine for all experimental sessions over the course of the day. The mean (SD) baseline pH of urine was fairly similar for the control (5.2 (0.2)), moderate (5.1 (0.2)) and vigorous (5.3 (0.2)) experimental sessions. Whereas, the baseline pH for light exercise was found to be lower (4.5 (2.0)) than control, moderate and vigorous. The urine pH levels for all experimental sessions decreased over the course of the day before increasing again. However, the time point at which this occurred varied for each experimental session. The earliest decrease in mean urine pH was found for control (4.2 (2.6)) and moderate exercise (4.7 (2.1)) between 9am - 12pm. This was followed by light (4.5 (2.0)) and vigorous (4.6 (2.0)) exercise decreasing between 12pm - 5pm. On the other hand, mean urine pH peaked the earliest for vigorous exercise (5.7 (0.3)) between 9am - 12pm, followed by control (5.5 (0.4)) between 12pm - 5pm and then light (5.4 (0.3)) and moderate (5.5 (0.3)) the latest between 5pm - 12am.

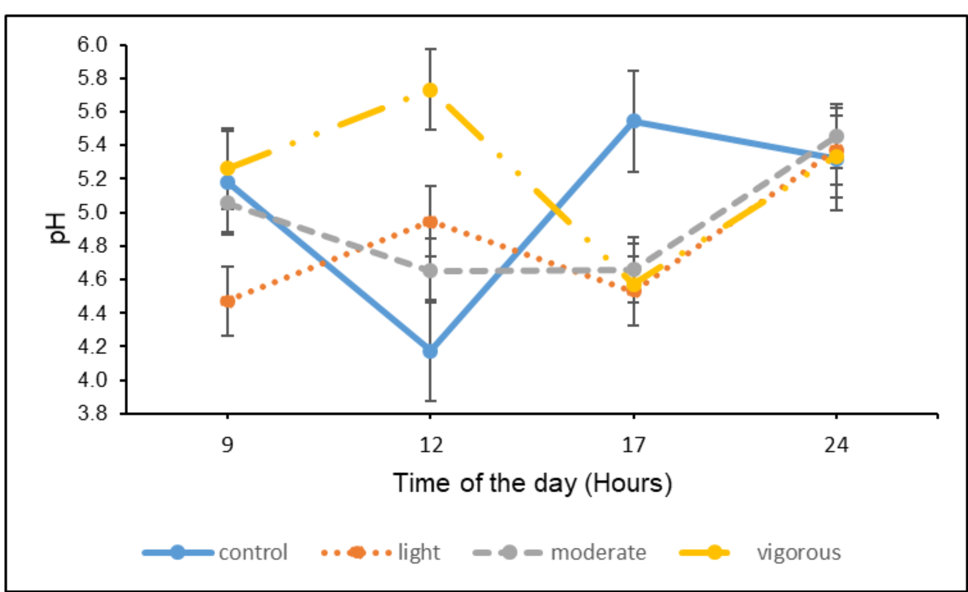


**Figure 6.7. Mean pH levels of the Urine samples throughout the day. Standard error is displayed as error bars on the graph. Key: Control – Blue; Light – Orange; Moderate – Grey and Vigorous – Yellow.**

### 6.4.3.3. Urinary fluoride excretion (UFE)

Tables 6.10 A to 6.10 D present the individual urinary fluoride excretion (UFE) and rate of UFE for control, light, moderate and vigorous experimental sessions. The values of individual UFE and rate of UFE for all sessions varied. Baseline fluoride excretion in urine ranged as follows: control session, 4.81 - 218.69 µg; light exercise, 32.03 - 442.88 µg; moderate, 3.25 - 121.19 µg and vigorous exercise, 107.35 - 323.50 µg. The baseline rate of UFE also varied for control (0.67 - 27.38 µg/hour), light (4.00 - 55.36 µg/hour), moderate (0.41 - 15.15 µg/hour) and vigorous (14.62 - 40.44 µg/hour).

The individual total 24-hour fluoride excretion also varied for control (145.70 - 1958.21 µg), light (93.54 - 1616.81 µg), moderate (247.38 - 1133.54 µg) and vigorous (276.26 - 1064.44 µg) experimental sessions. The individual total 24-hour rate of UFE ranged as follows: control session, 32.25 - 414.77 µg/hour; light exercise, 14.54 - 289.64 µg/hour; moderate exercise, 43.11 - 236.78 µg/hour and vigorous, 47.40 - 294.47 µg/hour.

**Table 6.10. Urinary F excretion (UFE: µg) and rate of UFE (µg/hour) for all subjects (n=8), individually, at pre- and post-fluoride ingestion.**

A. Individual urinary fluoride excretion for control experimental session.

| Participant | Time point | | | | | | | | Total 24 Hour | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12am till 9am (Baseline) | | 9am till 12pm | | 12pm till 5pm | | 5pm till 12am | | | |
| | Fluoride Excretion(µg) | Rate of UFE (µg/hour) | Fluoride Excretion (µg) | Rate of UFE (µg/hour) | Fluoride Excretion (µg) | Rate of UFE (µg/hour) | Fluoride Excretion (µg) | Rate of UFE (µg/hour) | Fluoride Excretion (µg) | Rate of UFE (µg/hour) |
| 1 | 136.49 | 17.06 | 140.99 | 35.25 | 286.31 | 57.26 | 87.61 | 12.52 | 651.40 | 122.09 |
| 2 | 218.69 | 27.34 | 1011.25 | 252.81 | 535.20 | 107.04 | 193.07 | 27.58 | 1958.21 | 414.77 |
| 4 | 214.44 | 26.80 | 180.20 | 45.05 | 51.68 | 10.34 | 396.68 | 56.67 | 843.00 | 138.86 |
| 5 | 5.36 | 0.67 | * | * | 263.32 | 35.11 | 127.75 | 18.25 | 396.43 | 54.03 |
| 6 | 7.40 | 14.80 | 231.48 | 77.16 | 63.84 | 12.77 | 24.30 | 3.47 | 327.02 | 108.20 |
| 9 | 45.83 | 5.73 | * | * | 310.53 | 62.11 | 36.78 | 5.25 | 393.14 | 73.09 |
| 10 | 4.81 | 4.81 | 33.19 | 8.30 | 65.69 | 13.14 | 42.01 | 6.00 | 145.70 | 32.25 |
| 11 | 54.77 | 6.85 | 219.14 | 54.78 | 135.71 | 27.14 | 134.34 | 19.19 | 543.95 | 107.96 |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

B. Individual urinary fluoride excretion for light experimental session.

| Participant | Time point | | | | | | | | Total 24 Hour | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12am till 9am (Baseline) | | 9am till 12pm | | 12pm till 5pm | | 5pm till 12am | | | |
| | Fluoride Excretion (µg) | Rate of UFE (µg/hr) | Fluoride Excretion (µg) | Rate of UFE (µg/hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg/hr) | Fluoride Excretion (µg) | Rate of UFE (µg/hr) |
| 1 | 442.88 | 55.36 | 260.41 | 86.80 | 297.10 | 59.42 | 616.42 | 88.06 | 1616.81 | 289.64 |
| 2 | 47.28 | 5.56 | 262.19 | 74.91 | 150.26 | 30.05 | 54.64 | 7.81 | 514.37 | 118.33 |
| 4 | 120.10 | 15.01 | 126.16 | 31.54 | 338.81 | 67.76 | 303.81 | 43.40 | 888.88 | 157.72 |
| 5 | 105.37 | 13.17 | 175.65 | 43.91 | * | * | 119.28 | 9.94 | 400.30 | 67.02 |
| 6 | * | * | 299.12 | 99.71 | 276.29 | 55.26 | 170.31 | 24.33 | 745.72 | 179.30 |
| 9 | 32.03 | 4.00 | * | * | 30.64 | 6.13 | 30.88 | 4.41 | 93.54 | 14.54 |
| 10 | * | * | * | * | * | * | * | * | * | * |
| 11 | 96.70 | 16.58 | 210.38 | 75.74 | 151.14 | 22.90 | 76.64 | 16.35 | 534.86 | 131.56 |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

C. Individual urinary fluoride excretion for moderate experimental session.

| Participant | Time point | | | | | | | | Total 24 Hour | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12am till 9am (Baseline) | | 9am till 12pm | | 12pm till 5pm | | 5pm till 12am | | | |
| | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) |
| 1 | 29.47 | 3.47 | 49.10 | 16.37 | 240.51 | 43.73 | 335.40 | 47.91 | 654.48 | 111.48 |
| 2 | 3.25 | 0.41 | 1.35 | 0.34 | 134.48 | 26.90 | 108.30 | 15.47 | 247.38 | 43.11 |
| 4 | 3.65 | 1.46 | 57.09 | 22.83 | 318.55 | 63.71 | 132.14 | 18.88 | 511.43 | 106.88 |
| 5 | 103.64 | 12.95 | * | * | 390.94 | 43.44 | 99.20 | 14.17 | 593.78 | 70.56 |
| 6 | 121.19 | 15.15 | 334.10 | 111.37 | 234.08 | 46.82 | 444.16 | 63.45 | 1133.54 | 236.78 |
| 9 | 37.39 | 4.67 | 41.96 | 13.99 | * | * | 222.73 | 31.82 | 302.08 | 50.48 |
| 10 | * | * | * | * | * | * | * | * | * | * |
| 11 | 82.49 | 9.82 | 232.57 | 25.84 | 138.95 | 33.35 | 112.46 | 25.10 | 566.47 | 94.11 |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

D. Individual urinary fluoride excretion for vigorous experimental session.

| Participant | Time point | | | | | | | | Total 24 Hour | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12am till 9am (Baseline) | | 9am till 12pm | | 12pm till 5pm | | 5pm till 12am | | | |
| | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) | Fluoride Excretion (µg) | Rate of UFE (µg /hr) |
| 1 | 109.39 | 16.09 | 86.54 | 18.13 | 28.62 | 5.72 | 51.71 | 7.46 | 276.26 | 47.40 |
| 2 | 116.93 | 14.62 | 107.25 | 26.81 | 50.26 | 10.05 | 142.82 | 20.40 | 417.26 | 71.89 |
| 4 | 323.50 | 40.44 | 371.76 | 92.94 | 192.33 | 38.47 | 176.86 | 25.27 | 1064.44 | 197.11 |
| 5 | 174.13 | 21.77 | 252.11 | 63.03 | * | * | 195.78 | 16.32 | 622.03 | 101.11 |
| 6 | 164.52 | 20.57 | 185.83 | 61.94 | 136.58 | 27.32 | 71.42 | 10.20 | 558.35 | 120.03 |
| 9 | * | * | * | * | * | * | * | * | * | * |
| 10 | 149.72 | 18.72 | 204.97 | 68.32 | 191.96 | 191.96 | 123.74 | 15.47 | 670.40 | 294.47 |
| 11 | 107.35 | 15.34 | 285.91 | 63.54 | 185.28 | 33.69 | 108.95 | 36.32 | 687.49 | 148.88 |

Note: * Blank sections in the table refer to missing data either from no sample for a given time point or due to the participant not having undertaken the experimental session.

Table 6.11 presents mean (SD) urinary fluoride excretion (UFE) and Table 6.12 presents mean (SD) UFE corrected for body weight, for all experimental sessions. The lowest mean UFE was found at baseline levels for control (1.1 (1.1) μg/kg bw), followed by light (1.5 (2.1) μg/kg bw) and moderate (0.8 (0.8) μg/kg bw) intensity sessions. The mean baseline UFE for vigorous exercise (2.0 (1.2) μg/kg bw) tend to be greater than control, light and moderate sessions.

Mean (SD) UFE for control, light and vigorous experimental sessions initially peaked between 9am - 12pm of 3.0 (3.9) μg/kg bw, 2.4 (1.8) μg/kg bw and 2.7 (1.7) μg/kg bw, respectively. A delay in peak UFE was observed by the moderate exercise session during 12pm – 5pm of 2.7 μg/kg bw.

**Table 6.11. Mean (SD) urinary fluoride excretion (UFE) (μg), for no (control), light, moderate and vigorous exercise.**

| Time point (Mean duration of collection: hour) | UFE (μg) | | | |
|---|---|---|---|---|
| | Control | Light | Moderate | Vigorous |
| 00:00 - 9:00 Baseline (9 hours) | 86.0 (91.4) | 140.7 (152.0) | 54.4 (47.9) | 163.6 (75.4) |
| 9:00 - 12:00 (3 hours) | 302.7 (354.4) | 222.3 (64.0) | 119.4 (132.4) | 213.5 (100.0) |
| 12:00 - 17:00 (5 hours) | 214.0 (167.8) | 207.4 (116.4) | 242.9 (100.3) | 130.8 (74.1) |
| 17:00 - 24:00 (7 hours) | 130.3 (122.2) | 196.0 (206.7) | 207.8 (134.7) | 124.5 (52.5) |

**Table 6.12. Mean (SD) urinary fluoride excretion (UFE), corrected for body weight (µg/ kg bw), for no (control), light, moderate and vigorous exercise.**

| Time point (Mean duration of collection: hour) | Body weight corrected UFE (µg / kg bw) | | | |
|---|---|---|---|---|
| | Control | Light | Moderate | Vigorous |
| 00:00 - 9:00 Baseline (9 hrs) | 1.1 (1.1) | 1.5 (2.1) | 0.8 (0.8) | 2.0 (1.2) |
| 9:00 - 12:00 (3 hrs) | 3.0 (3.9) | 2.4 (1.8) | 1.4 (2.1) | 2.7 (1.7) |
| 12:00 - 17:00 (5 hrs) | 3.0 (2.1) | 2.2 (1.9) | 2.7 (2.1) | 1.4 (1.3) |
| 17:00 - 24:00 (7 hrs) | 1.8 (1.5) | 2.5 (2.9) | 2.7 (2.3) | 1.6 (0.9) |

Table 6.13 presents the mean (SD) rate of UFE and baseline corrected mean (SD) rate of UFE for all experimental sessions. The baseline mean (SD) rate of UFE for moderate exercise (6.8 (5.8) µg/hr) tend to be lower compared to control (13.0 (10.2) µg/hr), light (15.7 (18.6) µg/hr) and vigorous (21.1 (9.0) µg/hr) experimental sessions. The baseline corrected mean rate of UFE for control (46.2 (76.2) µg/hr), light (37.9 (35.9) µg/hr) and vigorous (30.9 (22.3) µg/hr) sessions was found to peak between 9am - 12pm (3 hours post fluoride ingestion). However, there was a slight delay in the peak rate of UFE for moderate exercise (26.3 (21.4) µg/hr), occurring between 12pm - 5pm. The mean rate of UFE over a 24-hour period for all experimental sessions is illustrated by Figure 6.8.

**Table 6.13. Mean (SD) Rate of urinary fluoride excretion (UFE) (μg/hour), for no (control), light, moderate and vigorous exercise, also corrected for baseline (μg/hour).**

| Time point (Mean duration of collection: hour) | Rate of UFE (SD) (μg/hr) | | | |
|---|---|---|---|---|
| | Control | Light | Moderate | Vigorous |
| 00:00 - 9:00 Baseline (9 hrs) | 13.0 (10.2) | 15.7 (18.6) | 6.8 (5.8) | 21.1 (9.0) |
| 9:00 - 12:00 (3 hrs) | 59.2 (83.0) | 58.9 (35.2) | 27.2 (38.4) | 56.4 (25.6) |
| 12:00 - 17:00 (5 hrs) | 40.6 (33.4) | 34.5 (26.8) | 36.8 (19.9) | 43.9 (66.9) |
| 17:00 - 24:00 (7 hrs) | 18.6 (17.5) | 27.8 (29.7) | 31.0 (18.5) | 18.8 (9.8) |
| Total 24 hours | 131.4 (21.2) | 136.9 (18.2) | 101.9 (13.0) | 140.1 (18.2) |
| **Baseline corrected, Rate of UFE (SD) (μg/hr)** | | | | |
| 9:00 - 12:00 (3 hrs) | 46.2 (76.2) | 37.9 (35.9) | 17.9 (33.5) | 30.9 (22.3) |
| 12:00 - 17:00 (5 hrs) | 27.6 (31.6) | 16.5 (25.3) | 26.3 (21.4) | 20.0 (63.0) |
| 17:00 - 24:00 (7 hrs) | 5.6 (13.4) | 10.6 (15.1) | 21.1 (17.9) | -2.0 (11.3) |

Table 6.14 presents the percentage of total daily fluoride excretion for all sessions. In the control session, 50% of ingested fluoride was excreted in the first three hours post ingestion and 84% in the first eight hours post ingestion. The percentage of total daily fluoride excretion for light and vigorous exercise was similar to the control session. In the light exercise session, 49% was excreted in the first three hours and a total of 77% in the first eight hours. Vigorous exercise on the other hand, was found to excrete 47% fluoride in the first three hours and 84% in the first eight hours. The lowest percentage urinary fluoride excretion was found by the moderate exercise session, 29% in the first three hours with a total of 68% in the first eight hours post fluoride ingestion.

**Table 6.14. Percentage of total daily urinary fluoride excretion (UFE %), post fluoride ingestion, for control, light, moderate and vigorous experimental sessions.**

| Time point (Mean duration of collection: hour) | Percentage of total daily UFE (%) | | | |
|---|---|---|---|---|
| | Control | Light | Moderate | Vigorous |
| 9:00 - 12:00 (3 hrs) | 50 | 49 | 29 | 47 |
| 12:00 - 17:00 (5 hrs) | 34 | 28 | 39 | 37 |
| 17:00 - 24:00 (7 hrs) | 16 | 23 | 33 | 16 |

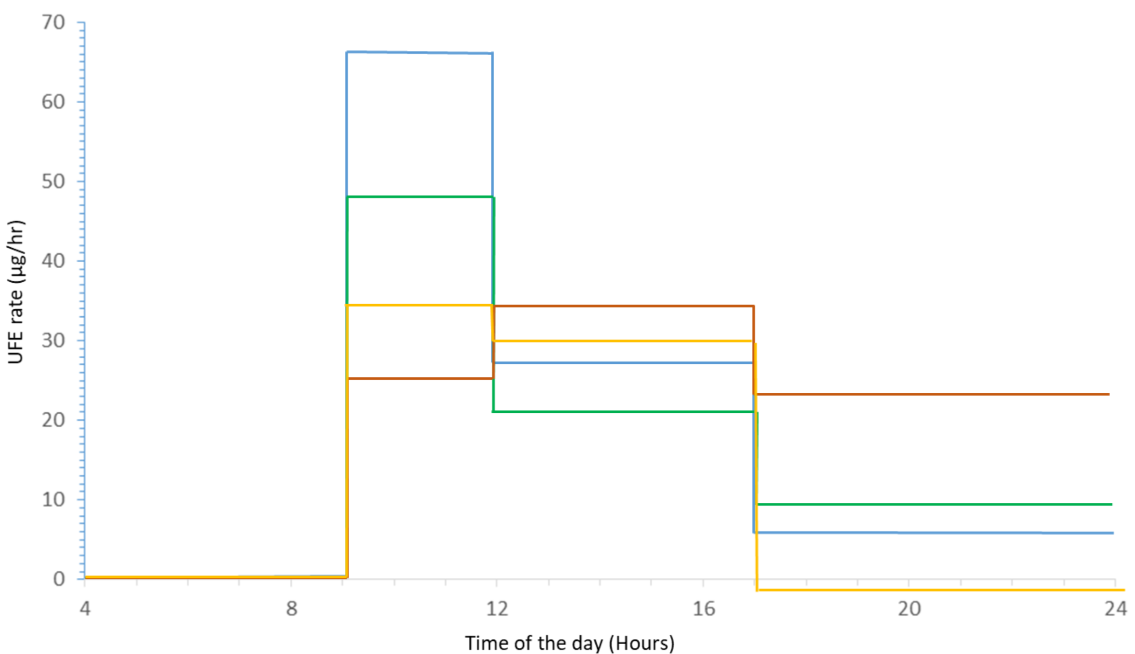


**Figure 6.8. Baseline corrected rate of urinary fluoride excretion (UFE) over a 24-hour period for no (control (blue line)), light (green line), moderate (brown line) and vigorous (yellow line).**

Figure 6.9 illustrates a comparison of the analysis of variation for rate of UFE (µg/hr) for 0 - 3 hours and Figure 6.10 for 3 - 5 hours, post fluoride ingestion for no (control), light, moderate and vigorous exercise sessions. No statistically significant differences were found for both time points ($p > 0.05$).

Figure 6.11 illustrates a comparison of the analysis of variation for rate of UFE (µg/hr) for 8 - 24 hours post fluoride ingestion for no (control), light, moderate and vigorous exercise sessions and a statistically significant difference was found for the moderate vs vigorous session ($p = 0.013$).

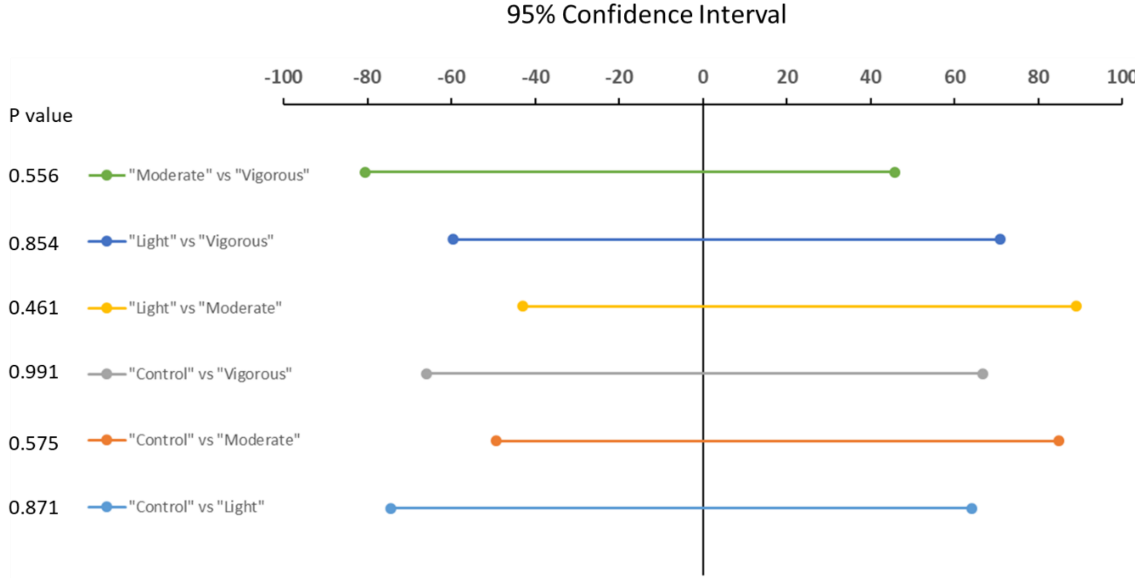


**Figure 6.9. Analysis of variation across all experimental sessions for rate of UFE (µg/hr) for 0 - 3 hours post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. 95% confidence interval of differences, lower and upper limit values have been reported.**

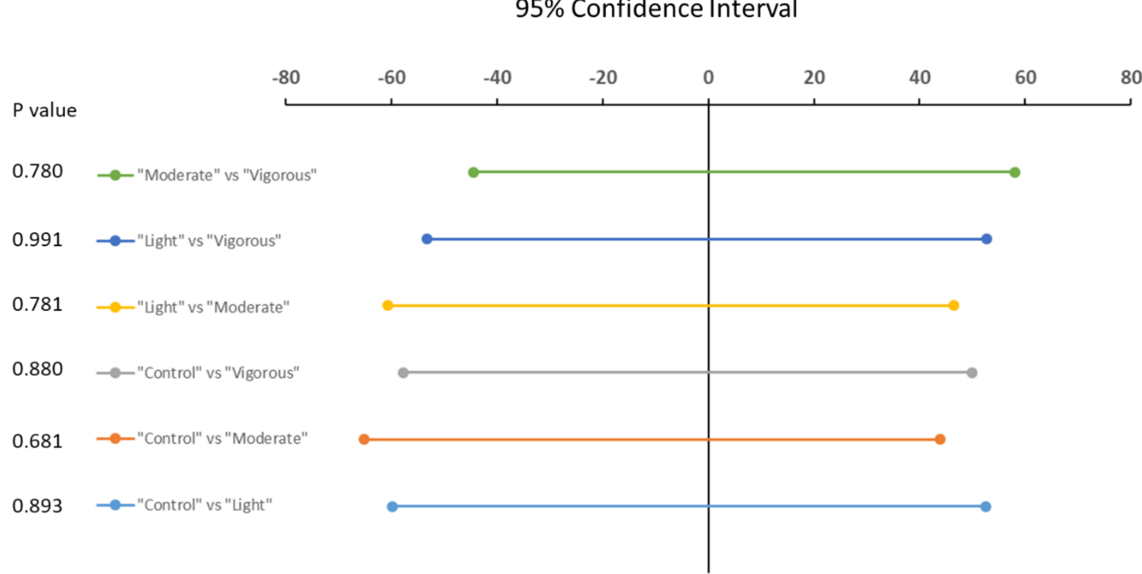


**Figure 6.10. Analysis of variation across all experimental sessions for rate of UFE (µg/hr) for 3 - 8 hours post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. 95% confidence interval of differences, lower and upper limit values have been reported.**

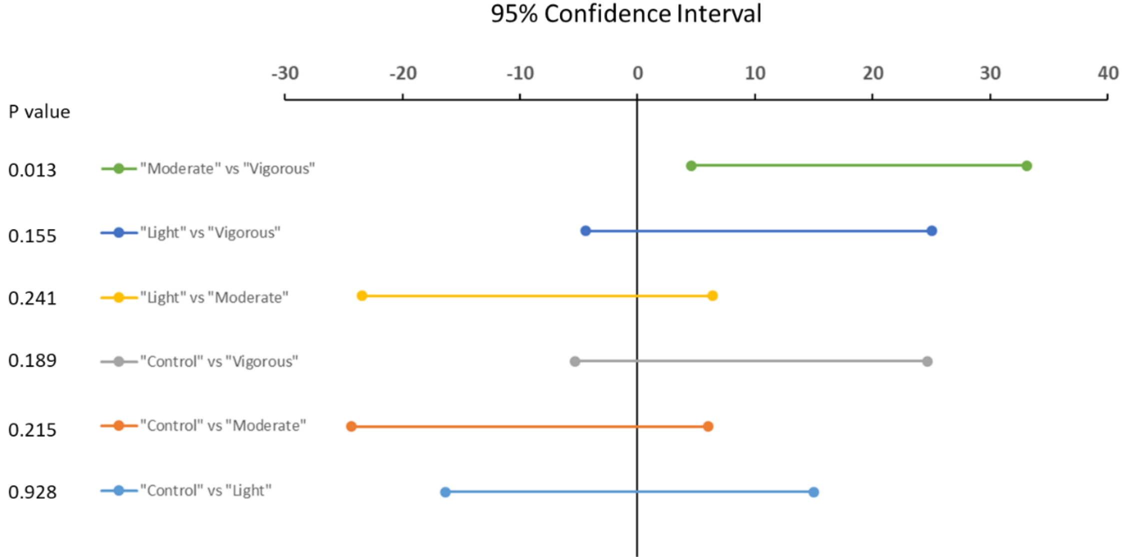


**Figure 6.11. Analysis of variation across all experimental sessions for rate of UFE (µg/hr) for 8 - 24 hours post fluoride ingestion. P values for all comparisons are illustrated beside the comparison. 95% confidence interval of differences, lower and upper limit values have been reported.**

## 6.5. Discussion

This section presents the discussion of the main study findings relating blood plasma and urine. Challenges relating to recruitment and aspects of health and safety along with missing data will also be discussed.

### 6.5.1. Plasma

#### 6.5.1.1. Pre-fluoride ingestion (baseline) plasma fluoride concentration

In the present study, the mean baseline plasma fluoride concentration for participants aged 18 to 35 years (n= 8) was lower for the control session compared to light, moderate and vigorous exercise sessions (Table 6.4). However, the mean baseline values for light, moderate and vigorous sessions in this study were all similar ranging from 36.9 - 45.2 ng/ml. The main study limitations were i) most participants performed the control session first due to health and safety reasons (as mentioned in section 6.5.7) followed by completing the three exercise intensities randomly; ii) the study was anticipated to be completed within 4 - 5 consecutive weeks for each individual. However, due to limited availability of the nurses, the 4 experimental sessions had to be arranged over the course of 2 - 3 months. Although participants were provided with more fluoride free toothpaste and repeatedly reminded to avoid fluoridated dentifrice, food and drinks (as listed in the participant information sheet) especially when travelling outside the Teesside area, there was no robust way of determining their adherence. Participants performed the control condition first (as discussed in section 6.5.7), therefore it is highly likely that their compliance with study instructions was greater during the first week or so compared to the following months. Thus, resulting in lower mean baseline values for the control condition. This may have also had an impact on the higher baseline values for plasma fluoride concentrations of the exercise sessions, as well as bone depletion. The higher mean baseline values for light, moderate and vigorous exercise sessions compared to the control session may also be explained by a net transfer of fluoride from bone surface to plasma due to a decrease in plasma fluoride concentrations overtime because of eliminating all other sources of fluoride intake and exposure (Cardosso *et al.*, 2008).

The overall mean baseline plasma fluoride concentration was 32.7 ng/ml, which was in line with the mean levels of 35.0 ng/ml reported for adults aged 10 - 38 years (Ekstrand, 1978) (Table 6.15). However, the participants were resident in areas with high fluoride levels i.e.

9.60 ppm, compared to this study (<0.3 ppm). Table 6.15 highlights the various mean plasma fluoride levels found in adults across the literature. A study by Cardosso *et al.* (2008) investigated plasma fluoride levels amongst five participants aged 27 - 33 years of both genders over a five-consecutive day period. Participants were on a low fluoride diet with fluoride free water (and toothpaste) during a thirty-day washout period and received a standardised low fluoride diet of <0.3 mg/day during the experimental period. Plasma levels were investigated by collecting blood samples at 8am, 11am, 2pm, 5pm and 8pm and mean plasma fluoride concentrations were found to range from 0.45 - 0.66 μmol/l (equivalent to 8 - 13 ng/ml).

The mean baseline level for the control condition in this study (i.e. 5.9 ng/ml, 0.31 μmol/l) is just below the levels found by Cardosso *et al.* (2008) and is indicative of the fluoride free regime prescribed to participants as part of the study exclusion requirements as shown by Table 6.6. Almost same plasma fluoride concentrations were found by the Sener *et al.* (2007) of 6 ng/ml who investigated plasma fluoride concentrations in women who had recently given birth (n= 125). The participants in the study of Sener *et al.* (2007) were a similar age range (20 - 30 years) to this study as well as having similar water fluoride concentrations (0.3 ppm) (Table 6.15).

The baseline values for control session are less than half the fasting (12 hour) plasma fluoride levels in children (n= 4) which were found to range between 13 - 21 ng/ml (Ekstrand *et al.,* 1980). This may be due to the steady bone fluoride dissociation rate of the age range of adults chosen for this study compared to children, as discussed in section 6.5.5.

**Table 6.15. Mean plasma fluoride levels in adults from literature and the current study**

| Age (years) | Fluoride concentration of water (ppm) | Mean Plasma fluoride level (ng/ml) | Author |
|---|---|---|---|
| 20 - 30 (n= 125) | 0.3 | 6.0 | Sener *et al.,* (2007) |
| 17 - 80 (n= 41) | * | 47.0 | Parkins *et al.* (1974) |
| 25 - 35 (n= 5)<br>25 - 35 (n= 5)<br>25 - 35 (n= 5) | 0.3<br>0.7<br>0.3 | 9.7<br>6.8<br>10.5 | Cardoso *et al.* (2006) |
| 27 - 33 (n= 5) | Low * | 10.0 | Cardoso *et al.* (2008) |
| 21 - 32 (n= 330) | * | 22.0 | Thomas *et al.* (2016) |
| 20 - 35 (n= 20) | 0.02 | 19.8 | Maguire *et al.* (2005) |
| 18 - 35 (n= 8) | < 0.3 | 32.7 | Current study |

Note: * no data has been provided on this section in the study

### 6.5.1.2. Post-fluoride ingestion plasma fluoride concentration

The baseline (pre-fluoride ingestion) level of plasma fluoride concentration was subtracted from the post-fluoride ingestion values to eliminate the background effect of fluoride in plasma. The lowest mean plasma fluoride concentration was found for the control session, ranging from 10.6 - 27.7 ng/ml, whereas, the greatest mean plasma fluoride concentrations was found for the moderate exercise session ranging from 168.3 - 226.2 ng/ml.

The mean peak plasma fluoride concentration for the control session (27.7 ng/ml) is in line with findings from Whitford *et al.* (2008) who found mean plasma fluoride concentrations in adults aged 24 - 32 years (n= 5) receiving naturally fluoridated water (0.85 ppm) to equal 28.3 ng/ml, following a dose of 0.33 mg fluoride. However, the dose of fluoride in this study was greater (2.2 mg NaF) but participants were resident in non-fluoridated areas (<0.3 ppm) compared to those in the study of Whitford *et al.* (2008) who received naturally fluoridated water.

Another study providing a similar dose (i.e. 2.00 mg fluoride) in adults aged 19 - 29 years (n= 4) found a mean peak plasma fluoride concentration of 106 ng/ml (Buzalaf *et al.,* 2008). This value is much higher than the mean plasma fluoride concentrations for the control session in this study (10.6 ng/ml - 27.7 ng/ml), however, are in line with the mean plasma fluoride concentrations for the light exercise session (64.8 ng/ml - 105.7 ng/ml).

Mean plasma fluoride concentrations for moderate and vigorous exercise ranged from 168.3 ng/l - 226.2 ng/ml and 51.1 ng/ml - 94.2 ng/ml, respectively. These are in line with findings from studies providing fluoride doses of 1.0, 1.5 and 10.0 mg with mean plasma fluoride concentrations of 51.1 - 52.6, 75 and 289 ng/ml, respectively (Oliveby *et al.,* 1989 [1,2,3]; Ekstrand *et al.*, 1989; Whitford, 1996). This may have arisen due to the enhanced absorption of fluoride following exercise.

The overall mean fluoride concentrations (Table 6.7) are higher for the exercise session compared to the control session which supports the correlation between exercise and plasma fluoride levels found by Zohoori *et al.* (2015) in a similar pilot study in humans. There has only been one study reporting the effects of exercise on fluoride metabolism in humans, so far. Zohoori *et al.* (2015) investigated the effects of exercise on plasma fluoride concentration, urinary fluoride excretion and fluoride renal clearance after the consumption of 1 mg fluoride tablet followed by no exercise and exercise (light, moderate and vigorous) conditions (n= 9). With respect to plasma fluoride, they found a rise in plasma concentrations with an increase in exercise intensity.

A few studies have reported the effects of exercise on fluoride metabolism in animals, as discussed in chapter 2. A recent study by Amaral *et al.* (2018) investigated the effect of chronic exercise on fluoride metabolism in fluorosis-susceptible mice exposed to high fluoride (n= 30), as well as exploring the relationship between fluoride concentrations in bone and plasma. The mice were divided into three groups: 1) no fluoride, no exercise; 2) 50 ppm fluoride, no exercise; and 3) 50 ppm fluoride, exercise. They found mean plasma and bone fluoride concentrations to be significantly higher in groups 2 and 3, compared to group 1 ($p < 0.001$ and $p < 0.01$, respectively), and concluded that chronic exercise had no effect on fluoride in plasma and bone. The findings from this study are in contrast with studies performed in animals.

Other studies performed in rats have found exercise to correlate with lower plasma fluoride levels. Basha and Sujita (2012) found exercise coupled with thermo-neutral temperatures to reduce fluoride toxicity in rats. Whitford (1989) found significantly lower and delayed peak

plasma fluoride levels in exercised rats compared to none exercised rats. This is like the findings from Lombarte *et al.* (2013) who suggested that the lower plasma fluoride levels in rats following exercise may be induced by an increased uptake of fluoride by bone. Although rats are primarily used to investigate fluoride metabolism in a laboratory setting due to their relatable fluoride pharmacokinetics (NRC, 2007), the findings from this study and a similar study performed in humans (Zohoori *et al.,* 2015) differ significantly. These differences may predominantly arise from inter-species variation or differences in protocol such as fluoride dose or exercise volume / intensity administered. Rats are known to metabolise fluoride around ten times faster than humans (Dunipace *et al*., 1995; Lombarte *et al*., 2013; Lima *et al*., 2014). Therefore, it is important to consider this whilst administering fluoride doses to humans for comparison. The fluoride dose in humans should be around four-five times greater than rats to gain similar fluoride levels in plasma (Dunipace *et al*., 1995).

Increasing the fluoride dose in humans to compare with rats may not always be ethical due to the very high fluoride doses used in rat studies. One example is a dose of 600 ppm used to specifically consider the effects of exercise on fluoride metabolism in rats (Basha and Sujita, 2012). More so, studies investigating fluoride pharmacokinetics in general have also used high doses of fluoride in rats e.g. 250 ppm (Collins *et al.,* 1995). Converting these doses of systemic fluoride ingestion for humans to compare the findings e.g. 600 ppm equivalent to 60 ppm or 60 mg/kg and 250 ppm equivalent to 25 ppm or 25 mg/kg, would be well above the safety threshold, thus posing an incredible risk of fluoride toxicity to humans. The World Health Organisation (2000) outlined single doses of 5 - 10 mg/kg body weight to cause acute toxic effects and ruled 70 - 140 mg/kg bodyweight as the lethal concentration range.

In rats, fluoridated water containing 25 - 100 ppm fluoride can elevate plasma fluoride concentrations to 3 - 10 μmol/l and induce dental fluorosis. In humans, fluorosis is known to be caused by similar plasma fluoride concentrations (3 - 10 μmol/l) and long-term ingestion of 1 - 10 ppm fluoride from drinking water can lead to plasma fluoride concentrations of 1 - 10 μmol/l (DenBesten and Li, 2011).

In this study, the highest mean plasma fluoride levels were found by the moderate exercise condition of 263.1 ng/ml, 13.85 μmol/l at 45 minutes post fluoride ingestion. This may have arisen from the increased blood circulation resulting from the increased cardiac output due to an increase in skeletal muscle activity (Manley, 1996). Increased blood circulation could increase the rate of fluoride absorption and distribution in soft tissues. Tomlinson *et al.*

(2012) demonstrated an increase in skeletal blood flow to cause an increase in fluoride metabolism.

The increase in lactate concentration following exercise may have also led to an increase in fluoride absorption resulting from a reduction in pH gradient which promotes the diffusion of fluoride (as HF) from extracellular to intracellular fluid (Buzalaf, 2011). Although fluoride tends to move from a low to high pH, this pathway may reverse in the muscles.

Manley (1996) also explained that the cardiac output following an increased work rate increases in an almost linear manner to meet the increasing oxygen demand but only up to the point where maximal capacity is reached. This may explain why higher mean plasma fluoride levels were achieved by the moderate condition compared to vigorous condition as participants may have reached their maximum potential during the vigorous condition. All participants reported extreme fatigue during the vigorous session which affected their ability to perform the condition in a consistent manner, many participants slowed down during the twenty-minute exercise session and required verbal encouragement to complete the condition.

#### 6.5.1.3. Pharmacokinetic data

The design of the study was based on the scientific method of pharmacokinetic analysis of the plasma fluoride concentration curve after intake of a single dose of fluoride which quantitatively describes the cumulative influence of the various metabolic processes and give important information about the kinetics of fluoride in the human body (Ekstrand *et al.*, 1990).

Since fluoride is already circulating in the human body it was necessary to take this into account and minimise variability in order to increase the possibility of detecting “exercise-related” differences. This was done by i) selecting a relatively narrow age range of 19 - 35 years and ii) correcting post-ingestion values for baseline, as discussed in section 6.5.1.2.

In this study, $C_{max}$ i.e. the maximum concentration of plasma was found to be greatest for moderate exercise (226.2 ng/ml), then light exercise (105.7 ng/ml) followed by vigorous exercise (94.2 ng/ml) and least for the no exercise (control) session (27.7 ng/ml). $C_{max}$ fluoride, for all sessions and $T_{max}$ i.e. time taken for $C_{max}$ to be reached, was reached within 30 - 60 minutes following ingestion which is in line with Whitford (1996) and Buzalaf *et al.*

(2011) who also reported that peak plasma fluoride levels were reached within 20 - 60 minutes following ingestion.

With respect to $T_{max}$, an increase was observed when the intensity of exercise increased. This led to a delay in peak fluoride concentration for the vigorous session. This may have possibly resulted from the gastric emptying of fluoride. Neufer *et al.* (1989) found gastric emptying to increase with increasing exercise intensities at 28% - 65% $VO_2$ max. However, they found a decrease in gastric emptying during high exercise intensities at 75% $VO_2$ max. The findings from this study along with exercise intensities used are in line with those of Neufer *et al.* (1989). Although Neufer *et al.* (1989) found such effects in treadmill running, a study by Rehrer *et al.* (1990) found similar results between running and cycling when investigating the gastric emptying of repeated drinking while running and bicycling for eighty minutes at 70% $VO_2$ max. With respect to fluoride, a reduced rate of fluoride absorption and slower rise in plasma fluoride concentration was accompanied by reduced gastric secretion in rats (Messer and Ophaug, 1991).

Mean AUC for no exercise (control) for the current study was 1584.0 ng.min.mil$^{-1}$. Mean AUC values (dose and baseline corrected) reported by Maguire *et al.* (2005) over 0 to 3 hours post ingestion of 500 ml naturally fluoridated soft, naturally fluoridated hard, artificially fluoridated soft, artificially fluoridated hard, and reference waters (containing fluoride ranging from 0.91 - 1.06 mg/l) ranged from 973 - 1217 ng.min.ml$^{-1}$ (n= 20). The mean AUC for control in this study was slightly above the levels reported by Maguire *et al.* (2005). AUC was calculated for 0 - 90 minutes post fluoride ingestion. Ninety minutes were chosen as the maximum duration of blood collection with respect to ethics and practicality for this study whilst also keeping in mind that plasma fluoride levels return to baseline levels within a few hours post ingestion, depending on the dose. Further plasma measurements are required to determine AUC with greater accuracy for this study.

AUC for all the exercise sessions ranged from 6025.2 - 14472.5 ng.min.ml$^{-1}$. The variation in AUC between subjects may have arisen from individual physiological differences in e.g. plasma volume, pH and volume of gastric secretions, gastro-intestinal motility and urinary pH (Shanbhag, 2008). Similarly, the variation in AUC between the exercise sessions may have resulted from the effect of different exercise intensities on the physiological functions which affect fluoride metabolism, as mentioned and summarised in table 6.16. In the present study, AUC was greatest for moderate exercise followed by light, vigorous and then control (no exercise). AUC 0 - 90 minutes post ingestion followed the same trend as shown

by $C_{max}$. Therefore, indicating that moderate exercise can increase the amount of fluoride absorbed along with its bioavailability.

## 6.5.2. Urine

### 6.5.2.1. Urinary flow rate

The mean urinary flow rate of each sample varied at different time points during the day for all experimental sessions ranging from 30.1 ml/hour to 91.7 ml/hour. This variation was likely due to the biological and lifestyle differences between each individual as well as dependant on the volume of fluids consumed and renal health (Robertson and Norgaard, 2002). Baseline mean urinary flow rate ranged from 30.1 - 55.6 ml/hour. Urinary flow rate is decreased during the night (Colwell, 2015) which explains why the range of urinary flow rates for baseline (12 am - 9 am) are lower compared to all other time points during the day.

Mean urinary flow rate for the control session (75.6 ml/hour) peaked in the first three hours immediately following exercise (9am - 12pm). This is in line with the normal resting rates of urine flow stated by Lamb and Gisolfi (1990), ranging from 48 - 90 ml/hour. Following light exercise, urine flow rate may remain unchanged or increase slightly (Buskirk and Puhl, 1996). This is supported by the findings from this study which found the highest mean peak in urine flow rate for the light exercise session of 91.7 ml/hour.

With respect to time, exercise led to a delay in peak urinary flow rate. Light (91.7 ml/hour), moderate (63.4 ml/hour) and vigorous (80.5 ml/hour) exercise all peaked, between 12pm - 5pm. This delay is likely caused by the reduction in renal blood flow during exercise which then reduces the glomerular filtration rate, along with increase in iso-osmotic reabsorption of water. Intense exercise is known to increase plasma antidiuretic hormone (Vasopressin) levels which also alter urine flow by increasing urine osmolarity and decreasing urine volume (Poortmans, 1984; Tipton, 2006). The level of increase in plasma antidiuretic hormone (Vasopressin) is dependent on the hydration levels of the exercising individual along with the ambient temperature and rate of fluid loss (Garrett and Kirkendall, 2000). Therefore, rate of urinary flow is directly proportional to the degree of hydration (Merrill, 2015).

During heavy or maximal exercise, glomerular filtration rate is expected to decrease 30% - 50% of the resting value and urine flow rate is decreased following moderate and vigorous

exercise (Buskirk and Puhl, 1996). However, the mean peak urinary flow rate for vigorous exercise (80.5 ml/hour) was higher than moderate exercise (63.4 ml/hour). This may have arisen from muscular fatigue which was reported by participants during the vigorous session. Fatigue affected their ability to complete the exercise at vigorous intensity for the whole duration of 20 minutes. Although, all participants completed the session, they did slow down at times and had to be encouraged to keep cycling at the level required. This may have affected the physiological responses produced following the vigorous session, including the urinary flow rate, vasoconstriction and glomerular filtration rate.

#### 6.5.2.2. Urine pH

The urinary pH levels for all experimental sessions decreased over the course of the day before increasing again at the end of the day. This may be diet-induced resulting from the consumption of acidic meals during the day which are known to reduce the pH of urine (Kanbara *et al.,* 2012). The time point at which this occurred however, varied for each experimental session. Control and moderate exercise experimental sessions had the lowest mean (SD) pH levels of 4.2 and 4.7 respectively between 9am - 12pm of the experimental day. Whereas, light and vigorous exercise experimental sessions found a decrease in their urinary pH of 4.5 and 4.6 occurring between 12pm - 5pm on the experimental day (excluding baseline).

In contrast, the time point of peak pH levels also varied, with the control experimental sessions mean urinary pH of 5.5 peaking between 12pm - 5pm. Light and moderate exercise had mean urinary pH peaks of 5.4 and 5.5 respectively between 5pm - 12am, whereas the mean urinary pH for the vigorous experimental session of 5.7 peaked the earliest, between 9am - 12pm. The sudden increase in urinary pH following vigorous exercise may have resulted from the increased production of carbon dioxide ($CO_2$) during aerobic and anaerobic metabolism along with the accumulation of lactate in the blood which converts $CO_2$ into bicarbonate ($HCO_{3-}$) and hydrogen ions (H+). Excessive $HCO_{3-}$ in the blood is then cleared through the urine and hence leading to an increase in urinary pH (Moriguchi *et al.*, 2004; Moriguchi *et al.*, 2002). This is supported by the findings of Moriguchi *et al.* (2004) who found urinary pH to increase abruptly thirty minutes following exercise on a cycle ergometer which continued to increase for the next two hours.

It is suggested that higher fluoride excretion is greatly influenced by more alkaline urine (Ekstrand *et al.*, 1980). Again, supported by the findings from this study which found

vigorous exercise (with the highest pH of 5.7) to have a mean urinary fluoride excretion (UFE) of 2.7 μg /kg bw during 9am - 12pm i.e. immediately after exercise. The greatest mean UFE was witnessed by the control session of 3.0 μg /kg bw during the 12pm - 5pm period with a pH of 5.5, which is amongst the high spectrum of pH's for this study.

#### 6.5.2.3. Pre-fluoride ingestion (baseline) urinary fluoride excretion (UFE)

The lowest mean UFE were found at baseline levels from all the collection time points for control (1.1 μg /kg bw), light (1.5 μg /kg bw) and moderate experimental sessions (0.8 μg /kg bw), as expected. Vigorous experimental session, however had the lowest UFE during the 12pm - 5pm time point of 1.4 μg /kg bw. Fasting conditions and elimination of fluoride sources from the diet and use in oral care during the wash-out period could be responsible for the low levels of urinary fluoride witnessed at baseline levels. Mean baseline values for urinary fluoride concentration in a similar study by Zohoori *et al.* (2015) ranged from 0.317 mg/l to 0.458 mg/l for adults aged 20 - 35 years, living in a non-fluoridated area.

#### 6.5.2.4. Post-fluoride ingestion urinary fluoride excretion (UFE)

Urinary fluoride excretion (UFE) values were corrected for 1) baseline and 2) body weight to obtain the levels due to ingestion of fluoride from the tablet provided in the study (i.e. eliminating existing fluoride levels in the body from unintentional ingestion or bone dissociation); and 2) to eliminate any effect arising from the varying body weight of each participant.

In this study, vigorous exercise displayed its peak mean fluoride excretion of 2.7 μg /kg bw the earliest, between 9am - 12pm. The control and moderate experimental session, on the other hand, peaked with a fluoride excretion of 3.0 μg /kg bw and 2.7 μg /kg bw, between 12pm - 5pm, respectively. The slightly later peak produced by the control experimental session may have resulted from the lack of physiological and metabolic burden exerted by exercise compared to rest experimental sessions (Nowrousian, 2010). However, the greatest delay in peak urinary fluoride excretion with the lowest peak of all four experimental sessions was observed by the light exercise intensity. A mean UFE of 2.5 μg / kg bw was excreted during 5pm - 12am of the experimental day. This again may be due to the lower metabolic burden exerted by light exercise compared to moderate and vigorous experimental sessions.

The percentage of total daily UFE (%) was greater during the first three hours post fluoride ingestion for the control (50%), light (49%) and vigorous (47%) exercise experimental sessions. This is supported by findings from Zipkin and Leone (1957) who found the greatest elimination of fluoride during the first hour following ingestion and 20% in the first three hours in rest experimental sessions. Whereas, for the moderate exercise experimental session, the percentage of total daily UFE (%) was greater for 3 - 8 hours post fluoride ingestion (39%). The decrease in blood flow and the increase in lactic acid concentration in the blood during moderate and vigorous exercise can reduce glomerular filtration and urinary pH (Freund, 1991). Therefore, the delay in UFE observed by moderate exercise may have been caused by the slightly later increase in pH caused by exercise. As mentioned previously, more alkaline urine favours fluoride excretion, therefore a delay in pH increase would have led to a delay in fluoride excretion.

Furthermore, the release of catecholamines during exercise would induce renal vasoconstriction and reduce muscular blood flow. The response of catecholamines is dependent on the intensity and duration of exercise (Garrett and Kirkendall, 2000). Renal vasoconstriction would also cause a reduction in fluoride excretion, as observed by the moderate exercise intensity.

The overall possible mechanisms following the physiological responses to exercise which may affect fluoride metabolism are summarised in table 6.16.

**Table 6.16. Primary mechanisms following physiological responses to exercise which may affect fluoride metabolism.**

| Tissue / Organ | Physiological adaptation to exercise | Possible mechanism affecting fluoride metabolism | Author (year) |
|---|---|---|---|
| Muscle | Increased lactate concentration | Reduction in pH gradient will promote the diffusion of fluoride (as HF) from extracellular to intracellular fluid, thus, increasing fluoride absorption. | Buzalaf (2011) |
| Vascular system | Increased blood circulation | Increased blood circulation could increase the rate of fluoride absorption and distribution in soft tissues.<br>Increased blood circulation could also increase uptake by bone | Tomlinson *et al.* (2012) |
| Stomach | Increased Gastric emptying following light to moderate intensity exercise.<br>Decreased gastric emptying following high intensity exercise | Reduced gastric secretion has been found to reduce rate of fluoride absorption and slow the rise in plasma fluoride concentrations in rats. | Neufer *et al.* (1989)<br>Rehrer *et al.* (1990)<br>Messer and Ophaug (1991) |
| Kidneys | Decreased glomerular filtration rate | Decreased glomerular filtration and decreased urine flow rate could lead to a reduction in fluoride excretion. | Buskirk and Puhl (1996) |
| | Increase in urinary pH following vigorous exercise | More alkaline urine is known to increase fluoride excretion. | Ekstrand *et al.* (1980) |

| | | | |
|---|---|---|---|
| | Renal vasoconstriction | Renal vasoconstriction could reduce fluoride excretion | Garrett and Kirkendall (2000). |

### 6.5.3. Exercise

The type of exercise activity chosen for this study was a cycle ergometer. The main advantages for using a cycle ergometer in this study protocol arose from greater practicality and health and safety. It is easier to collect samples and monitor participant health and safety during stationary cycling on a cycle ergometer compared to treadmill running (Jones and Poole, 2013). Cycle ergometer also lowers the risk of falling during exercise.

Although, it is known that both cycle ergometer and treadmill exercise are equally effective in improving functional exercise capacity (Polen and Joshi, 2014). The limitations however include the greater effect of muscular adaptation to cycling over time and greater impairment of ventilation compared to running. Still, running leads to central fatigue and decreased maximal strength compared to exercise on a cycle ergometer (Millet *et al.,* 2009).

In this study, participants were mostly comfortable performing the cycle ergometer exercise during light and moderate intensities. The vigorous intensity however, was a lot more challenging. Many participants reported fatigue within the first 5 - 10 minutes and struggled to sustain pedalling at the required rate for vigorous intensity for the required duration of 20 minutes. In such cases, participants were repeatedly verbally motivated to keep going to complete the exercise and all of them successfully completed it. However, further work may be required to determine a shorter duration of exercise sufficient to produce the physiological responses required to affect fluoride metabolism. This would help minimise fatigue and consequently increase adherence to the protocol for future studies.

### 6.5.4. Recruitment

Strategy, challenges and success of participant recruitment for this project are presented here. Since the recruitment of relevant staff (nurses) for sample collection had a great impact on the overall participant recruitment, the aspects of staff recruitment are also discussed.

#### 6.5.4.1. Participant

Of the approximate 2,396 staff and 18,576 students (full time and part time) that were contacted, only 13 participants agreed to take part of which 8 participants aged 18 to 35 (4 male and 4 female) completed the study. This age range was chosen to exclude any

physiological differences resulting from age and both genders were included in equal numbers to eliminate any gender bias.

Participant recruitment started in May 2016. To begin with emails were circulated within the Health and Social Care Institute and School of Health and Social Care staff and students but with a poor response rate, permission was gained from deans of other schools in Teesside University to contact their students. Upon successfully gaining permission, school managers circulated the recruitment material within their respective Schools. An advertisement was also displayed in the Health and Social Care institute newsletter.

Of the handful of people who showed interest from across the whole university, five were recruited, of which only four took part in the experimental sessions. One participant completed the pre-experimental session but later dropped out due to work commitments. Other respondents were originally interested in the study but did not provide consent to take part.

Three main barriers to recruitment were apparent by responses from participants. Firstly, the protocol involved blood collection and many participants were afraid or phobic to needles or blood. Secondly, the study was time consuming requiring participants to attend on five occasions for one hour for the first session (pre-experimental) and three hours each for the remaining four sessions (experimental sessions). This was followed by collecting 24-hour urine samples. Many participants were unable to commit the time required due to work or family commitments. Lastly, the highly extensive recruitment inclusion/exclusion criteria invalidated the eligibility of those who were interested. For example, the restricted postcodes meant that those who lived outside the Teesside area were unable to take part and individuals with any medical condition or medication were also unable to take part. This affected a few participants who would have otherwise liked to participate.

Whilst considering the extensive demands of the study which included time commitment, blood and urine collection as well as the study exclusion requirements i.e. not using any fluoridated dentifrice or consuming food and drink high in fluoride during the wash-out and whole experimental period, a monetary incentive of £100 in Love2Shop gift vouchers was included as part of the recruitment strategy. Although it influenced and favoured interest in those who participated, it still yielded a poor response and recruitment rate. Another point to consider was that recruitment took place during the summer period when many individuals were away on holiday. Following the holiday season, study and work

commenced for most of the target population i.e. staff and students of Teesside University, which would have had an impact on the response rate.

Due to poor recruitment, an ethical amendment was made to include ‘family and friends’ of staff and students at Teesside University as part of the target population. After successfully gaining ethical re-approval the recruitment material was circulated again, and a further five participants were recruited of which one had to be excluded due to health and safety (refer to section 6.5.7). The remaining four participants completed all or part of the study. Participant recruitment was ended in November/December 2016 due to the limited availability of the nurses who were involved in blood collection for the study, as well as that of the researcher. This is further explained in the next section,

#### 6.5.4.2. Staff

A nurse or phlebotomist was required to perform the venous blood sampling at various time points according to the study protocol. However, there were many challenges faced with regards to recruitment for this study. Finding qualified and trained nurses registered with the Nursing and Midwifery Council (NMC) from the local Teesside area with availability during weekday working hours was proving to be difficult. The majority of nurses were employed and had work commitments during the time the study was anticipated to be undertaken.

Following no luck with recruitment of a nurse, a recruitment agency arranged for a small group of certified and trained phlebotomists to cover blood sampling for the experimental sessions. Again, it was difficult to find a single individual who was available to commit to the study, therefore, a group of 4 phlebotomists agreed to cover various experimental sessions. However, during the first experimental session of the study with three participants present, the phlebotomist was faced with difficulty in successfully withdrawing blood samples. Challenges included inaccurately locating the required vein, unable to collecting multiple samples and issues with blood clotting in the blood collection system midway during the collection period. The participants also reported that they experienced discomfort during and following the blood sampling. The participants doubted the competency of the phlebotomist and felt uncomfortable to allow him/her to undertake blood collection for the remaining of the study. Therefore, the recruitment agency was contacted to explain the situation with regards to unsuccessful blood sampling and the contract to hire their

phlebotomists was ended whilst keeping participant health and safety in mind. This as a consequence made some potential participants withdraw from the study.

Eventually, two dental nurses from the Dental Hospital, School of Dental Sciences at Newcastle University were recruited. Both dental nurses were fully trained and experienced in blood collection. They were able to successfully collect multiple blood samples using cannulation and participants were also comfortable with the blood collection. However, the only limitations were that with no budget to employ a full-time nurse for a fixed period of time, the nurses had to travel from Newcastle for every session which was time consuming and dependant on traffic and weather conditions. In situations when the nurses were delayed in arriving, participants had to wait to start their session which was discouraging for the participants. Also, the nurses had agreed to visit Teesside University to assist with the project on their days off work which were a combined of two weekdays amongst themselves, this limited the availability that could be offered to participants which was a limitation from the perspective of participant recruitment. More importantly, the availability of the nurses was limited from August 2016 till December 2016 which meant that data collection could only continue according to their availability. On the other hand, the researcher was conducting this study as part of a 3-year full time PhD programme, expected to end in December 2017. This not just limited but also restricted participant recruitment until December 2016 upon which the study had to discontinue in order to allow enough time for sample and data analysis, and write-up of the study findings.

### 6.5.5. Participant age

The age of participants eligible to be recruited for this study was 18 to 35-year olds. This age range was chosen to ensure the inclusion of individuals whose skeletal bone density was still increasing with a net positive flow of fluoride ions from plasma to bone. Long bones stop growing in length around the age of 18 years in females and 20 years in males (Mahan and Raymond, 2016), but peak bone mass is reached around the age of 25 to 35 years (Henderson *et al.,* 2008).

Therefore, the chosen age range avoided other age ranges such as children in whom bones are increasing in density at a higher rate with a much greater net movement of fluoride into bone from plasma, and older people in whom there is an overall equilibrium between the net movement of fluoride between bone and plasma (Maguire *et al.,* 2005). The relatively

narrow age range (18 - 35 years) was also chosen to minimise variability in order to increase the possibility of detecting condition (exercise)-related differences.

Overall, this age group gave a more typical picture of the majority of the population, while having the advantage of a group in which the net flow of fluoride ions from plasma to bone is still positive. Plasma fluoride levels are influenced by the relative rate of bone accretion and dissolution, so the choice of this age range minimised variability in order to increase the possibility of detecting “treatment-related” differences. Unfortunately, the size of the study did not allow a comparison between genders to be made. The relatively invasive nature of the study, requiring cannulation and plasma sampling also restricted the size of the study and the age group which could be studied. The development and validation of suitable biomarkers as a substitute for plasma sampling would facilitate the study of larger populations as well as younger and older age groups than those studied here.

### 6.5.6. Consumables

Blood lactate measurements were originally undertaken in the beginning of the study. A finger pin prick test was used to collect blood lactate measurements at 3, 5 and 7 minutes’ post exercise. If peak lactate was not reached after 7 minutes (determined by a drop of lactate value in the subsequent sample) another measurement was taken after 2 minutes. Blood was collected into a 25 µl capillary tube for injection into a Yellow Springs lactate analyser (YSI model 2300; YSI, Inc., Yellow Springs, OH) calibrated prior to each test according to the manufacturer's specifications and standards. However, the lactate analyser failed to produce results for every blood sample that was inserted into the machine. The analyser displayed a technical error and a replacement analyser was unable to be sourced within the duration of the study. This led to the loss of quantifiable blood lactate data in the beginning of the study, upon which this measurement was excluded from the study protocol due to technical practicalities.

### 6.5.7. Health and Safety

It was highly important to consider participant health and safety during the experimental sessions. The greatest risk arose from the use of cannula / blood sampling and from the exercise.

It was originally proposed that participants would undertake each experimental session randomly according to the Latin's square design. However, during recruitment when participants were given the chance to ask any questions, it was reported that a participant had previously fallen queasy and unconscious during the use of a cannula in a similar situation. Therefore, to avoid any such incident with any of the participants (as some had never experienced the use of a cannula previously) it was decided that the participants would perform their control experimental session first followed by the exercise experimental sessions randomly. This would minimise any risk associated with performing exercise on a cycle ergometer in the case of any light-headedness or ill health due to the use of a cannula. Nurses performing the blood sampling were also at hand to provide appropriate care / assistance in any such circumstances.

This was in fact experienced by participant number 8 who reported feeling light-headed upon the collection of the first blood sample. The nurses provided appropriate care in the first instance to help the participant recover as the participant insisted on completing the session. However, after 30 minutes of resting flat on the ground, the participant still felt unstable, it was decided to remove the cannula and eliminate the participant from the remainder of the study in the best interest of the participant's health and safety. The nurses thoroughly examined the individual and found no ill health. They explained from experience how it is common for some individuals to feel queasy upon the sight of needles or blood as a psychological response.

No further incidents regarding health and safety were experienced for the remainder of the study.

### 6.5.8. Missing Data

Five of the 8 participants completed all five sessions. The remainder 3 participants only completed part of the sessions. This was due to change in circumstances of participant availability. During the recruitment period, all participants confirmed availability for all the five sessions, however due to the experimental phase extending further than the originally proposed month (in accordance to nurse availability) some participants started study / work and therefore were unable to complete all study sessions which resulted in the loss of data for certain exercise intensities. Data for blood and urine samples at certain time points was also lost in the case when further blood samples were unable to be extracted and when participants forgot to collect or did not feel the need to urinate during certain hours. Table 6.17 provides an overview of the missing data.

**Table 6.17. An overview of the missing data across the study.**

| Participant | Missing Data | Reason |
|---|---|---|
| 1 | None | |
| 2 | Control session | Phlebotomist unable to take bloods, participant unable to repeat control session when dental nurses were recruited. |
| 4 | None | |
| 5 | None | |
| 6 | Urine Sample: midnight – 9am for light session | Participant forgot to collect sample. |
| 9 | A. Blood sample for control session: 90 mins post fluoride ingestion<br>B. Vigorous session<br>C. Urine Samples:<br>9am – 12pm for control and light session<br>12pm – 5pm for moderate session | A. Blood started clotting in the cannula, no blood could be collected even after flushing the cannula with saline.<br>B. Session schedule clashed with participant's study timetable therefore unable to attend the vigorous session.<br>C. Participant didn't need to urinate during these hours. |
| 10 | Light Session and Moderate Session | Participant had to leave the Teesside area for a few weeks due to unforeseen circumstances. |
| 11 | None | |

### 6.5.9. Sample Preparation and Analysis

Challenges were faced during the preparation and analysis of blood samples. One challenge arisen from the volume of blood samples. A 5 ml blood sample was taken from each participant at each session/time which is expected to produce around 1.5 - 2.5 ml of plasma upon centrifugation. Due to physiological differences, hydration levels, etc. A between- as well as within-individual variation in the plasma volume was observed. This led to different volumes of plasma available for triplicate analysis of fluoride. The exact sample weight was measured using a bench scale and used during data analysis.

On a few occasions, part of (triplicate) samples were lost during the HMDS acid diffusion method. In such situations, the lost sample was noted, and duplicate analysis was performed.

## 6.6. Summary of findings

The main findings of the study are summarised as below:

- The mean peak urinary flow rate ranged from 63.4 ml/hour to 91.7 ml/hour for all sessions. There was a delay in urinary flow rate for the exercise sessions compared to the no exercise (control) session.
- The mean peak Urinary Fluoride Excretion (UFE) ranged between 2.4 µg/kg bw and 3.0 µg/kg bw, for all sessions. A delay in peak UFE was observed by the moderate exercise session (2.4 µg/kg bw) compared to all other exercise and no exercise session.
- The mean peak rate of UFE ranged from 36.8 µg/hr to 59.2 µg/hr, for all sessions. The rate of UFE was also reduced and delayed for the moderate exercise session (36.8 µg/hr) compared to all other exercise and no exercise session.
- The amount of fluoride excreted in urine over the first 3 hours following fluoride ingestion as a percentage of total fluoride excretion ranged from 47% to 50% for no exercise, light and vigorous exercise. A reduction in percentage urinary fluoride excretion over the first 3 hours was found by the moderate exercise session of 29%.
- The amount of fluoride excreted in urine over the first 8 hours following fluoride ingestion as a percentage of total fluoride excretion ranged from 68% to 84%. The

lowest percentage urinary fluoride excretion over the first 8 hours was again found by the moderate exercise session of 68%.

- The time to reach maximum plasma concentration of fluoride ($T_{max}$) ranged from 44 minutes to 50 minutes for all sessions. A delay in $T_{max}$ was observed by the vigorous exercise session at 50 minutes. However, no statistically significant differences in $T_{max}$ were found between all sessions ($p > 0.05$).
- The baseline-corrected peak plasma concentration of fluoride ($C_{max}$) ranged between 27.7 ng/ml - 226.2 ng/ml. The greatest $C_{max}$ was found by the moderate exercise session (226.2 ng/ml). Statistically significant differences in $C_{max}$ were found between control and moderate ($p < 0.001$) and light and moderate ($p = 0.010$). No other statistically significant differences in $C_{max}$ for the remaining sessions were found.
- The baseline-corrected area under the curve for 0 to 90 minutes (AUC (0-90)) following fluoride ingestion ranged from 1584.0 $ng.min.ml^{-1}$ - 14472.5 $ng.min.ml^{-1}$ for all session. Statistically significant differences in AUC were found between control and all three exercise sessions (light, moderate and vigorous) ($p < 0.05$); whereas there was no statistically significant difference in AUC between light and vigorous exercise ($p = 0.222$).

## 6.7. Overall conclusion and Future work

Findings from this study indicate that moderate exercise can increase plasma fluoride levels and lead to a delay in UFE in adults. Although, this study was performed in a laboratory setting and physiological responses of every day physical activity on fluoride metabolism may vary. This study must be performed with a larger number of participants to confirm such effects in the wider population. Other structures of exercise can also be investigated e.g. the effects of ‘acute vs chronic’, ‘continuous vs intermittent’ exercise and ‘active vs sedentary’ participants.

Considering the important role of lactate and blood pH in exercise and its potential effects on fluoride metabolism, it is suggested that future studies should try to measure lactate or blood pH to look into the association of lactate concentration and fluoride absorption.

Since, the age group at most risk of excessive fluoride retention are young children, further work is also required to determine the effects of exercise on fluoride metabolism in young children. These effects will most likely differ from adults due to the different physiological responses following exercise in children compared to adults. Findings can help provide an evidence base for stakeholders and decision makers in dental public health as well as health professionals who may wish to review the fluoride dose and time of administration.

# Chapter 7. Study 4: Development of a practical and ethically approved protocol to investigate the effects of exercise on fluoride excretion in children.

## 7.1. Introduction

As discussed in chapter 2, the prevalence of dental caries amongst young children is a global public health problem. In England, 12% of three-year-olds and 30% of five-year-olds are affected by Dental Caries (Public Health England (PHE)[A], 2014; PHE, 2016). Public health initiatives such as fluoridated milk schemes have been rolled out across schools, in several countries including the UK, in order to tackle the problem. However, previous research has indicated that the current UK milk fluoridation scheme does not provide adequate protection for the prevention of dental caries (Ketley and Lennon, 2000).

Considering the practicality and ethical issues surrounding the methods to measure fluoride absorption and excretion, especially in relation to blood collection, chapter 6 investigated the effects of exercise on fluoride metabolism in adults. The data gained was used to design a study protocol to investigate the effects of exercise on fluoride metabolism in children. This chapter presents the development of a protocol which is suitable and practical to perform with children, along with ensuring it is ethical by gaining ethical approval. The developed protocol was also piloted to assess its practicality. This chapter presents the successes and challenges faced in the pilot study.

## 7.2. Overall aims and objectives

### 7.2.1. Aims

The overall aim was to develop a feasible and ethically sound protocol for an experimental study on the effects of exercise on fluoride excretion in children.

### 7.2.2. Objectives

The objectives in order to achieve the study aim were as follows:

- To develop a protocol framework to assess the effects of exercise on fluoride metabolism in children.
- To develop a protocol that is ethically sound.
- To pilot the child project in order to assess the feasibility of the protocol.

## 7.3. Development of the protocol

### 7.3.1. Initial development of the protocol

**Design**: The initial study was designed as a two-treatment repeated measures cross-over study with child subjects in a field setting with semi-structured playground conditions. The study was proposed to take place over two sessions of the experiment separated by a washout period of at least a week.

1) Fluoridated tooth brushing, no exercise (control)
2) Fluoridated tooth brushing, playground activity.

The study would be conducted at the same time of day for each experiment for each subject, to control for circadian rhythms.

**Recruitment**:

The protocol proposes to recruit 30 participants aged between 5 - 7 years recruited from primary schools in non-fluoridated areas (e.g. in Teesside).

Contact details of all local primary schools could be obtained from the council website. The headteacher of the schools are to be contacted via email / writing i.e. an invitation letter will be handed in at the reception desk first followed by a phone call if no response is gained

within 2 weeks, and permission is to be obtained to distribute research information to parents along with consent forms / PAR-Q, asking parent / guardians to confirm their child/children meet the inclusion criteria. Participant Information sheets are to be distributed amongst the children and assent is to be gained using the assent form before the experiment.

Parents who will consent for their children to take part are to complete the consent form and return them to school by the deadline indicated on the consent forms. Followed by this the children with parental consent will be provided with child information sheets and assent forms to indicate they will like to take part.

Participant Inclusion criteria:

- Healthy child aged between 5 and 7 years, defined by a satisfactory Pre-Activity Readiness Questionnaire (2012) (PAR-Q).
- No history of acid-base disturbance and not receiving a therapeutic diet
- Live in a non-fluoride postcode area for at least 3 months prior to and during the study

Participant Exclusion criteria:

- Any symptoms or known presence of cardiovascular, respiratory or metabolic disease (such as high blood pressure, asthma or diabetes) have any musculoskeletal problems;
- Lactose intolerant
- Exercise restrictions
- Taking any recreational drugs, herbal, homeopathic or any alternative remedies in the last week prior to participation, are taking any prescribed medication
- Home postcode indicates the fluoride concentration in the area is above 0.3mg/litre.

**Pre-experimental Session:**

The lead researcher (Maria Sajjad) will visit the school on the morning of the experimental session to obtain urine samples collected by parents from the previous night for evaluation of baseline measures along with the height/weight measurements in a private area with the help of the school nurse. Participants will then be informed on the layout of the experimental session i.e. semi-structured playground activity.

**Experimental Session:**

Study Exclusion Requirements:

In order to eliminate / minimise fluoride intake / exposure from all other sources during the study, participants will be set a fluoride-free regime. They will be provided with a fluoride-free toothpaste to use along with being asked to refrain from the following, the day before as well during the whole experimental day and washout period:

- Avoid using any toothpaste, mouthwash containing fluoride or any other significant fluoride products such as fluoride tablets and fluoride supplements
- Avoid drinking tap water or using tap water in coffee, soups etc. if the Teesside area (TS1 – TS26 and TS29) is left (e.g. to visit a friend outside these postcodes).
- Avoid drinking tea
- Avoid all other types of fish apart from saithe and cod fillet. Not more than 500g per week of saithe and cod fillet, will be allowed to be consumed. However, these should be avoided for at least 48-hours prior to the experiment day.

The experimental session is to be carried out at the primary school where children will be recruited from. The study would be conducted around 10am / during the mid-morning break for each experiment and participant. The lead researcher (Maria Sajjad) will visit the school in the morning and familiarise the children with the structure of the experimental session. The children will then brush their teeth around 10am with fluoridated toothpaste in accordance with the 'Tooth Brushing Programme' recommended by the Oral Health foundation. Child participants will then either read a book/watch a video (control session) or undertake a semi-structured game of tag in the playground (experimental session) for 10 minutes. This will be followed by obtaining 24-hour pooled urine samples at timely intervals with the help of the school nurse / teachers whilst at school and parents / guardians whilst

at home. The two experimental sessions will be separated by a “wash-out period” of at least 1 week.

Heart rate will be continually monitored during and 10 minutes after exercise.

**Sample collection:**

Urine will be collected by spontaneous voiding during the following time intervals:

1. One pooled urine sample from 9pm before the experimental day up until about 10:00 am just before tooth brushing.
2. One pooled urine sample from 10:00 am to 06:00 pm during the experimental day.
3. One pooled urine sample from 06:00pm to 12:00am during the experimental day.
4. One pooled sample from 12:00am to 8am the next day.

**Sample transportation, preparation, storage and analysis:**

**Urine:** All urine samples will be transported to Teesside University by the researcher (Maria Sajjad). Collected urine samples would be stored in a refrigerator upon collection till analysis. The volume and pH of each pooled urine samples would be measured within 24 - 48 hours of collection and 3 x 10 ml aliquot would be taken, and the rest would be disposed by flushing down a designated toilet which would be cleaned by Virkon solution. The samples would then be analysed for fluoride immediately using the direct fluoride ion electrode selective method – as described in chapter 4 (Martinez-Mier *et al.,* 2011).

To thank the parents for their time and assistance in the urine collection at home, they would be offered £15 Love 2 Shop gift vouchers as an incentive for each session (total £30), per child.

### 7.3.2. Applying for ethical clearance

To check that the protocol is ethical, four attempts / submissions for ethical approval were made to the Research Governance and Ethics Committee, School of Health and Social Care, Teesside University from December 2016 to May 2017. The ethics application with the original protocol (Appendix 4) was submitted in December 2016 and the response gained from the ethics committee was to revise the application and re-submit. Members of the committee held serious reservations about the ethical propriety and risk benefit balance of the proposed study, at this point.

A re-submission with a response letter addressing the comments (letter attached, Appendix 5) was made in February 2017. This gained a response of: provisionally approved subject to amendments (chair's action). The committee required a few additional documents to support the application along with more information regarding recruitment and recruitment material, in particular. The comments provided were addressed (letter attached, Appendix 6) and a revised application was re-submitted in April 2017. The chair of the ethics committee yet provided further comments in an email (response email attached, Appendix 7) regarding the use of fluoridated and non-fluoridated toothpaste, which were addressed with a further revised ethics application in May 2017.

This project was finally approved by the Research Governance and Ethics Committee, (Reference number 206/16 - approval attached – Appendix 8) in May 2017 (final ethics application attached with supporting documents, Appendix 9).

### 7.3.3. Revision of the protocol

Several revisions of the protocol were made following comments from the ethics committee. Below are some of the main amendments:

- The study design was modified to undertake the study in a laboratory setting (Teesside University) as well as the originally proposed field setting (primary schools).
- The recruitment method was amended to include two recruitment routes (this included a modification of the original recruitment method).
- The recruitment and study information material was also amended accordingly, and new material was created to include as part of the recruitment and study information material (attached in Appendix 9).
- The exercise sessions would be led by co-supervisor (Dr Liane Azevedo) instead of researcher (Maria).
- Risk assessments for the exercise were included.
- The method insured child stigmatisation was avoided, especially in the school setting where not all children would be participating.
- Sample collection from children and recording of baseline measures would no longer rely on help from school teachers and school nurses. The researcher (Maria) and

co-supervisor (Dr Liane Azevedo) would now be responsible for collecting these measures.

- All health and safety procedures were put into place for urine sample collection, storage and transport.
- Risks and benefits of eliminating fluoridated toothpaste and using non-fluoridated toothpaste were clearly identified.
- Insurance approval for the project was gained from the University's insurers (UMAL).

### 7.3.4. Finalising the protocol

The protocol was developed, and several revisions were made according to comments from the ethics committee before the below protocol was finalised.

**Design**: This study is designed as a two-treatment repeated measures cross-over study with child subjects in a field / laboratory setting with semi-structured playground conditions. The study is proposed to take place over one week which will include two sessions of the experiment separated by a 3-day washout period. The study is to be conducted at the same time of day for each experiment for each subject, to control for circadian rhythms.

1) Fluoridated tooth brushing, no exercise (control)
2) Fluoridated tooth brushing, playground activity.

**Recruitment**: Participants are to be recruited via two routes. Around 30 children in total are anticipated to be recruited.

1) From primary schools in non-fluoridated areas (e.g. in Teesside).
2) Through staff and students of Teesside University and their family and friends.

- **Route 1: Primary schools**

Contact details of all local primary schools are to be obtained from the council website. The head teacher of the schools are to be contacted via email / letter which will be handed along with a response form in at the reception desk followed by a phone call if no response is gained within 2 weeks. Head teachers are to be provided with an invitation letter and consent form for class teachers. Permission will be obtained to distribute a research information pack to parents consisting of an invitation letter to parents with an attached

information sheet, a child participant information sheet for the parents to go through with their children, A PAR-Q which will be used to ask parents to confirm their child/children meet the inclusion criteria. Parents will use this to screen their child's eligibility and their child can only take part if they are free from any of the stated health risk factors in the PAR-Q. They will also be given consent forms to complete and assent forms to be completed by their child at this stage.

The researcher (Maria Sajjad) will also visit the school to give parents, children and teachers the opportunity to ask any questions. Completed consent and assent forms / PAR-Q are to be returned directly to the researcher (Maria) or via the school teacher.

- **Route 2: Teesside University**

Children of all staff and students of Teesside University and their family and friends will be eligible to take part. All staff/students are to be made aware of the project through an email sent by the researcher (Maria Sajjad) via the school administrator. The research information pack consisting of the following will be attached to the email: a parent information sheet, a child participant information sheet for the parents to go through with their children, A PAR-Q asking parents to confirm their child/children meet the inclusion criteria (their child will only be able to take part if they are free from any of the stated health risk factors in the PAR-Q). They will also be given consent forms to complete and assent forms to be completed by their child at this stage.

An item will also be displayed in the Health and Social Care Institute newsletter to advertise the study. The information pack and newsletter advert will include contact details of the researcher (Maria) for any questions. Parents will have the option to arrange a meeting with the researcher (Maria) during working hours/lunch hour at the University if they wish to meet to discuss their queries. Any parent and child who will like to take part in the study will be asked to complete the attached consent and assent form / PAR-Q and email it to the researcher (Maria).

In order to be eligible to participate, the participants will have to meet the inclusion and exclusion criteria, described in section 7.3.1., along with the additional exclusion criteria:

Participant Exclusion criteria:

- Wear nappies / pyjama pants
- Attend any pre- or post-school activities

**Pre-experimental Session:**

Route 1: Primary Schools - The lead researcher (Maria Sajjad) and co-supervisor (Dr Liane Azevedo) will visit the school on the morning of the experimental session.

Route 2: Teesside University - Participants will be invited to attend a pre-experimental session at the physiology laboratory at Teesside University on the morning of the experimental session.

Urine samples collected by parents from the previous night will be obtained for evaluation of baseline measures along with the height/weight measurements. Height and weight will be recorded by the researcher (Maria Sajjad) in the presence of project co-supervisor (Dr Liane Azevedo), using a portable scale and measuring tape. This will take place in a private area 1) provided by the school or 2) at the physiology laboratory, Teesside University. Minimal / no disrobing of the children would be required i.e. no coats and jackets or shoes, all other clothing would be permitted. Participants will then be informed on the layout of the experimental session i.e. of the semi-structured playground activity.

Dr Liane Azevedo, Senior Lecturer in Physical Activity and Public Health, Certified Exercise Physiologist (EP-C) by the American College of Sports Medicine (ACSM), with PhD in Exercise Science and DBS checked will lead and supervise the exercise session.

**Experimental Session:**

Study Exclusion Requirements:

In order to eliminate / minimise fluoride intake / exposure from all other sources during the study, participants will be set a fluoride-free regime. They will be provided with a fluoride-free toothpaste to use along with being asked to refrain from the following, the day before as well during the whole experimental day:

- Avoid using any toothpaste, mouthwash containing fluoride or any other significant fluoride products such as fluoride tablets and fluoride supplements

- Avoid drinking tap water or using tap water in coffee, soups etc. if the Teesside area (TS1 – TS26 and TS29) is left (e.g. to visit a friend outside these postcodes).
- Avoid drinking tea
- Avoid all other types of fish apart from saithe and cod fillet. Not more than 500g per week of saithe and cod fillet, will be allowed to be consumed. However, these should be avoided for at least 48-hours prior to the experiment day.

Route 1: Primary Schools – participants will be given the option to perform the study at their primary school around 10 am during playtime (mid-morning break) or they could visit the physiology lab at Teesside University with their parents on a week day during the school holidays.

Route 2: Teesside University – participants will be invited to visit the University at 10 am to perform their sessions on a week day during the school holidays.

The child participants will brush their teeth using fluoridated toothpaste (Colgate Kids Junior 6+ toothpaste containing 1450 ppm NaF). The toothbrushing will take place in accordance with the ‘Tooth Brushing Programme’ recommended by the Oral Health foundation (included in Appendix 9). Child participants will either read a book/watch a video provided by their class teacher (control session) or undertake a semi-structured game of tag in the playground (experimental session) for 10 minutes after performing a 5-minute warm up consisting of stretches and light exercises.

Children will be asked to wear a heart rate monitor RS400 (Polar Electro Oy, Kempele, Finland). The researcher and co-supervisor will demonstrate on themselves how to place the HR monitor. If the elastic belt needed adjusting, they will ask the child to remove the belt, adjust it and give it back to the child. Heart rate (HR) will be continually monitored by the researcher during and 10 minutes after exercise to ensure the children are exercising at the intensity level required. Children will be asked to exercise at vigorous intensity. The children’s heart rate is required to stay between 70 to 85% of maximum heart rate (HR max). Physical activity will be discontinued if the child reaches maximal heart rate above 85% of their maximum heart rate (220-age).

Each heart rate monitor will be set at the child’s target heart rate zone (70 to 85% of HR max). If the child’s heart rate falls under or above the target heart rate zone (70 to 85% HR max) the watch will beep and the researchers (Maria Sajjad and Dr Liane Azevedo) will

verbally encourage children to increase the intensity (if below HR zone); or will ask the children to slow down to allow heart rate to recover (if above HR zone).

**Sample collection:**

**<u>Urine</u>**: Pooled urine samples will be collected by the researcher (using urine collection hats) whilst the children are at school / University and by parents / guardians whilst at home (a complete urine collection guide will be provided to parents). At any time if the child participant passes faeces and urine at the same time, the sample (faeces and urine) will be disposed in the toilet and recorded as missing/not applicable/etc. Urine samples will be collected at the following time points.

1) One pooled urine sample from 09:00 pm before the experimental day up until about 10:00 am just before tooth brushing.
2) One pooled urine sample from 10:00 am to 06:00 pm during the experimental day.
3) One pooled urine sample from 06:00 pm to 12:00 am during the experimental day.
4) One pooled sample from 12:00 am to 08:00 am the next day.

**Sample transportation, preparation, storage and analysis:**

**<u>Urine</u>:** All urine samples will be transported to Teesside University by the researcher (Maria Sajjad). The samples will be safely stored in tightly closed plastic collection bottles, sealed with Para film and transported in polystyrene boxes / cool bags. Collected urine samples will be stored in a refrigerator upon collection till analysis.

The volume and pH of each pooled urine samples will be measured within 24 – 48 hours of collection and 3 x 10 ml aliquot would be taken, and the rest would be disposed by flushing down a designated toilet which would be cleaned by Virkon solution. The samples will then be analysed for fluoride immediately using the direct fluoride ion electrode selective method – as described in chapter 4 (Martinez-Mier *et al.,* 2011).

To thank the parents for their time and assistance in the urine collection at home, they would be offered £15 Love 2 Shop gift vouchers as an incentive for each session (total £30), per child.

## 7.4. Piloting the protocol

Following successfully gaining ethical approval, it was decided to pilot the study to test the practicality of recruitment and data collection involving child participants. Ethical approval was gained in May 2017 and because the local primary schools were approaching the end of summer term followed by school closure due to summer holidays, it was decided to approach recruitment via route 2: Teesside University. Recruitment for this study was only undertaken between June 2017 and September 2017 due to time constraints of the PhD completion time. Recruitment emails and material were circulated amongst staff and students in the Health and Social Care Institute, Teesside University, on multiple occasions.

Six parents (with six children in total) responded with interest in the study. Two of the children did not meet the inclusion criteria. Three parents were happy for their children to take part; however, they were unable to attend the University in the summer holidays due to family commitments and other plans. Only one parent (project co-supervisor) and their child (male, aged 6 years) provided assent / consent and took part in the pilot study.

The study was performed according to the protocol (section 7.3.1). The layout of the pre-experimental and experimental sessions was feasible and conducted with ease. The parent reported one missing urine sample from the pooled baseline sample (night before experimental session) due to the child wetting the bed. Apart from that no problem was reported by the parent in urine collection for the child for both study sessions.

The child participant reported feeling comfortable throughout the study and enjoyed the experience of participating in research. No problems were reported during urine sample collection by the child. As, the study was only piloted with one child to assess the practicality of the protocol, sample and data analysis was not performed on the collected urine samples.

During the control session, the child had to be encouraged to remain seated on multiple occasions and stay engaged in the video for 10 minutes. The parent informed the researcher that her child is a very active child and has a very short attention span which may be the reason why the child had to be encouraged to remain seated. However, this may not be the case with every child and the study must be performed with more children to be able to assess the practicality with greater reliability.

On the other hand, during the experimental session, Dr Azevedo, leading the exercise found that 10 minutes of the same structure of activity i.e. game of tag, led to boredom after 3 - 5

minutes which impacted the intensity of exercise the child was performing at (confirmed by the measured heart rate). Dr Azevedo suggested using two different types of games/activities at vigorous intensity for 5 minutes each, one immediately after the other. She recommended this would lead to greater engagement by the children in the activity and hence greater performance at the exercise intensity required. These points should be kept in mind to revise the protocol slightly to conduct a larger pilot investigating the effects of exercise on fluoride metabolism in children.

## 7.5. Discussion and Conclusion

Gaining ethical approval for a protocol designed to investigate the effects of exercise on fluoride metabolism in children was found to be extremely challenging. Upon gaining ethical approval, recruitment of child participants to pilot the study lead to another challenge. A longer recruitment period and wider advertising might be required to recruit more participants. The study must be performed with a few more participants in one session, recruited from both recruitment routes: 1) Primary Schools and 2) Teesside University. The child showed good acceptance of the exercise routine and outcome measures. However, a more diverse exercise protocol might be more engaging for the child. Finally, there is a need to explore the practicality of performing such a study in a field setting.

# Chapter 8. Overall discussion

## 8.1. Introduction

As discussed in chapters 2 and 6, dental caries remains a substantial global public health problem. In England, 12% of three-year-olds and 30% of five-year-olds are affected by dental caries (Public Health England (PHE)[A], 2014; PHE, 2016). The latest number of hospital procedures for children aged 18 and under to have multiple teeth removed were 42,911 in the year 2016-2017, costing the NHS in excess of £36 million (Telegraph, 2018).

The preventative role of fluoride in dental caries has long been established. Exposures to low levels of fluoride have been shown to reduce dental caries by 50% (Welbury *et al.,* 2012). Many factors affect the metabolism of fluoride including exercise. Knowledge around fluoride metabolism is limited and there is a major lack of understanding relating the effects of exercise on fluoride metabolism within the human body. Therefore, further understanding the mechanisms of fluoride metabolism may help to maximise the beneficial effects of fluoride for caries prevention, while minimising the risk of dental fluorosis.

The methods to measure fluoride absorption and excretion which provide an indication of fluoride metabolism often involve practicality and ethical issues, especially in relation to blood collection in young children. Therefore, this project firstly aimed to initially assess the use of Dry Tips and Lashley cups for the collection of ductal saliva for studies on fluoride metabolism in humans (Study 1 – chapter 4). Secondly, it aimed to assess and compare practical methods of blood and saliva collection for studies on fluoride metabolism in humans (Study 2 – chapter 5). Ultimately, this project aimed to investigate the effect of different intensities of continuous exercise on fluoride metabolic responses in adults (Study 3 – chapter 6). The generated data, in the adult study, were used to design a study protocol for potential studies on the effects of exercise on fluoride metabolism in children (Study 4 – chapter 7).

## 8.2. Overall method

### 8.2.1. Study 1: Practicality and suitability of 'Dry Tips' and 'Lashley Cups' for the collection of saliva: A Preliminary Investigation.

In this study the endogenous fluoride contents of Dry Tips were investigated. Three preparation methods were tested: 1) Direct Ashing, 2) NaOH as an ashing aid, and 3) No ashing. The ashed and un-ashed Dry Tip samples were then analysed using the Hexamethyldisiloxane (HMDS) acid-diffusion method (Martinez-Mier *et al.*, 2011). In part of this study, Dry Tips were used to collect ductal saliva samples from 25 human participants.

Lashley cups manufactured at Teesside University were tested at three different levels on 5 participants in total by: 1) A dentist experienced in collecting ductal saliva at Bauru, Brazil; 2) A dental therapist from the Dental School at Teesside University, Middlesbrough; and 3) An experienced dental nurse from the Dental Hospital, Newcastle University, Newcastle.

### 8.2.2. Study 2: Determination of fluoride in saliva and plasma: sample collection and analysis.

This study was divided into two phases (A and B). Phase A involved the collection of 1) venous whole blood, 2) venous plasma and 3) capillary whole blood, from 15 human participants. Phase B involved the collection of 1) whole saliva, 2) sublingual-submandibular saliva and 3) venous plasma, from 25 human participants. All samples were collected from participants in non-fasting condition, following their usual diet and routine of tooth brushing. The Plasma samples were analysed using the HMDS acid-diffusion method (Martinez-Mier *et al.,* 2011). The saliva samples were analysed using the direct fluoride ion electrode selective method (Martinez-Mier *et al.,* 2011).

### 8.2.3. Study 3: The effects of different intensities of continuous exercise on fluoride metabolism in adult humans.

This experiment was designed as a four-treatment, repeated measures cross-over study. During a pre-experimental session, participant's height and weight were measured and a $VO_2$ peak test was performed to guide the three exercise intensities (light, moderate and vigorous) for the experimental sessions.

For the experimental sessions, participants were invited to attend the laboratory at 9:00 am on each experiment day in fasting conditions when the first blood sample was collected. The participants were then provided with a low fluoride breakfast. After breakfast, a polar heart rate monitor belt was worn on the chest. They were then given one 2.2 mg sodium-fluoride tablet (Endekay Fluotabs 6 years+) with 250ml low fluoride bottled water (≤0.08 μgF/ml). Participants then either rested (control session) or undertook the exercise (experimental sessions) at approximately 9:30am.

Participants warmed up for five minutes at a self-selected speed before initiating the exercise on the cycle ergometers for twenty minutes at one of the following intensities; light, moderate and vigorous intensities (determined at the pre-experimental session). Each experimental session was separated by a "wash-out period" of at least one week. Heart rate was continually monitored during and 10 minutes after exercise. Blood samples (5 ml) were collected 0, 30, 45, 60 and 90 minutes post fluoride ingestion. Twenty-four-hour urine was also collected by spontaneous voiding. The Plasma samples were analysed using the HMDS acid-diffusion method (Martinez-Mier *et al.,* 2011). The urine samples were analysed directly using the fluoride ion selective electrode (Martinez-Mier *et al.,* 2011).

### 8.2.4. Study 4: Development of a practical and ethically approved protocol for potential studies on the effects of exercise on fluoride excretion in children.

An initial protocol was developed to assess the feasibility and ethical acceptance of a protocol for a prospective study on the effects of exercise on fluoride excretion in children. The step by step protocol was based on the four-treatment, repeated measures cross over study in adults (study 3 - chapter 6). Four attempts / submissions were made to gain ethical approval. The protocol was revised based on comments from the ethics committee and the project was finally approved. Following successfully gaining ethical approval, the protocol was piloted to test the practicality of recruitment and data collection involving child participants.

## 8.3. Overall results

### 8.3.1. Study 1: Practicality and suitability of Dry Tips and Lashley Cups for the collection of saliva: A Preliminary Investigation.

The Dry Tips were successfully ashed using the muffle furnace. However, ashing of the Dry Tip samples with ashing aid was not as successful as those without ashing aid. Fairly high concentrations of fluoride were detected from the blank Dry Tips, regardless of the method used. The results were inconsistent and unreliable across the ashed and un-ashed samples.

The dentist in Brazil performing the saliva collection reported successful saliva collection using the Lashley cup produced at Teesside University. However, the collected samples were unable to be transported to the UK for fluoride analysis. Back in the UK, there were many challenges faced in the collection of ductal saliva by the dental therapists at Teesside University and dental nurses at Newcastle Dental Hospital. Several attempts were made to locate the duct and collect saliva. However, the dental team were not able to collect any ductal saliva samples, using the Lashley cups, from the volunteers.

### 8.3.2. Study 2: Determination of fluoride in saliva and plasma: sample collection and analysis.

**Phase A:** The collection of capillary and venous whole blood is both practical and can be performed with ease using trained staff. Capillary blood was found to be an unsuitable indicator of fluoride absorption levels. There was a significant positive correlation (Pearson correlation = 0.692, $p = 0.027$) between venous whole blood and venous plasma, with a ratio of 0.65. Venous plasma was found to be the most suitable biomarker for measuring fluoride absorption levels.

**Phase B:** The collection of whole saliva and SL-SM saliva was collected successfully with ease using the drool / spit (without trained staff) and oral syringe method (with trained staff), respectively. Both methods were deemed practical from a collection perspective. There was a statistically significant positive correlation (Pearson correlation = 0.410, $p = 0.053$) between fluoride concentrations in whole saliva and SL-SM saliva. SL-SM saliva was considered as the most preferred measure of fluoride absorption from these preliminary findings.

A slight positive non-statistically significant correlation, was found between venous plasma and whole saliva (Pearson correlation = 0.048, p = 0.832). Whereas, a slight negative non-statistically significant correlation was found between venous plasma and SL-SM saliva (Pearson correlation = -0.047, p = 0.836).

A mean ratio of 1.30 was found between whole saliva and venous plasma and 0.18 between SL-SM saliva and venous plasma. Overall, plasma was considered as the most preferred measure of fluoride absorption levels for studies on fluoride metabolism in humans.

### 8.3.3. Study 3: The effects of different intensities of continuous exercise on fluoride metabolism in adult humans.

Exercise was found to cause a delay in urinary flow rate compared to the no exercise session. A delay in peak urinary fluoride excretion (UFE) and rate of UFE was observed by the moderate exercise session of 2.7 µg/kg bw and 26.3 µg/hr (between 12pm - 5pm), respectively, compared to all other exercise (light (2.4 µg/kg bw, 37.9 µg/hr) and vigorous (2.7 µg/kg bw, 30.9 µg/hr)) and no exercise (3.0 µg/kg bw, 46.2 µg/hr) session which peaked between 9am - 12 pm.

The lowest percentage UFE over the first 8 hours post fluoride ingestion was also found by the moderate exercise session of 68%, compared to no (84%), light (77%) and vigorous (84%) exercise. No statistically significant differences were found for the analysis of variation for rate of UFE (µg/hr) for 0 - 3 hours and 3 - 5 hours, post fluoride ingestion (p value >0.05) for no (control), light, moderate and vigorous exercise sessions.

With respect to plasma, the greatest maximum plasma fluoride concentration ($C_{max}$) was again found by moderate exercise at 45 minutes post fluoride ingestion of 226.2±115.6 ng/ml, compared to no (23.6±14.3 ng/ml), light (102.7±58.2 ng/ml) and vigorous (94.2±58.1 ng/ml) exercise. However, a delay in time to reach Maximum Plasma Concentration of fluoride ($T_{max}$) was observed by the vigorous exercise session at 50 minutes post fluoride ingestion.

Statistically significant mean (95% CI) differences were found in $C_{max}$ between control and moderate, (-246.2, -325.2 to -167.3) (p = 0.001) and light and moderate (-110.2, -200.3 to - 20.1) (p = 0.010) exercise.

Statistically significant differences in AUC were found between control and all three exercise sessions (light, moderate and vigorous) ($p < 0.05$); whereas there was no statistically significant difference in AUC between light and vigorous exercise ($p = 0.222$).

### 8.3.4. Study 4: Development of a practical and ethically approved protocol to investigate the effects of exercise on fluoride excretion in children.

Following ethical approval, the protocol was piloted. Only Six parents (with six children in total) responded with interest in the study. Following the recruitment procedure, only one parent and their child (male, aged 6 years) provided assent / consent and took part in the pilot study.

The study was successfully conducted according to the developed protocol. The parent reported one missing urine sample from the pooled baseline due to the child wetting the bed. Apart from that no problem was reported by the parent in urine collection for the child for both study sessions. The child participant reported feeling comfortable throughout the study and enjoyed the experience of participating in research. No problems were reported during urine sample collection by the child. As, the aim was to pilot the practicality of recruitment and data/sample collection to refine the protocol, sample and data analysis was not performed on the collected urine samples.

Although, the study must be performed with more children to be able to assess the practicality with greater reliability. Following the pilot study, it was discussed that using two different types of games / activities at vigorous intensity for 5 minutes each, one immediately after the other may lead to greater engagement by the children in the activity and hence greater performance at the exercise intensity required. These points would be kept in mind to revise the protocol slightly to conduct a larger pilot investigating the effects of exercise on fluoride metabolism in children.

## 8.4. Overall discussion

In summary, the study rationale arose from the failure of the current UK milk fluoridation scheme in providing adequate protection against dental caries in young children (Ketley and Lennon, 2000). Since the fluoridated milk part of the UK milk fluoridation scheme is provided during the mid-morning, before playtime, exercise was considered to play a role in the metabolism of fluoride. However, there is a lack of understanding on the effects of exercise on fluoride pharmacokinetics, especially in humans. Investigating the effects of exercise on fluoride metabolism in children would pose many practical and ethical challenges, therefore, the main aim of this project was to investigate the effects of different intensities of continuous exercise on fluoride metabolic responses in adults (Study 3 – Chapter 6).

Fluoride absorption levels are most commonly measured using blood plasma samples. The collection of blood is invasive, unethical and poses the risk of harm, especially when involving young children. Ductal saliva levels are also indicative of fluoride absorption and have a direct relation to fluoride in plasma. The current methods required to measure ductal saliva are invasive. Therefore, due to the necessity of finding easy and inexpensive methods for the collection of ductal saliva, Study 1 (Chapter 4) aimed to assess and develop the use of Dry Tips (commercially available) and Lashley cups (previously used; Fukushima *et al.,* 2011) for the collection and determination of fluoride in ductal saliva.

This study found Dry Tips to contain high endogenous fluoride as well as failing to recover fluoride which made them unsuitable for the use in studies involving fluoride analysis. However, ductal saliva samples can still be collected in a less invasive manner which may benefit researchers and practitioners looking into the collection of ductal saliva for other research and clinical purposes.

On the other hand, failure in saliva collection using the Lashley cups arose from the requirement of staff with specialist training to correctly identify the parotid duct. Considering the implications in locating the parotid duct, the use of Lashley cups may not be favourable in the wider population, due to individual anatomical variations of the mouth as well as the need for a trained dentist to perform the sampling.

Due to unsuccessful collection of ductal saliva using the Lashley cups, alternative methods for ductal saliva collection were considered. SL-SM saliva also has a direct relation to plasma; therefore, the collection of SL-SM saliva was considered for Study 2 (Chapter 5).

In Study 2A, the collection of capillary whole blood and venous whole blood were found to be simple to collect (with the help of trained staff for venous whole blood). However, due to high fluoride concentrations in capillary whole blood, it was disregarded as a method of collection for studies on fluoride metabolism.

A correlation was found between venous whole blood and venous plasma. Although, analysing whole blood doesn't require any sample preparation and saves time, money and resources, it often requires to be analysed immediately. Immediate sample analysis is a huge burden and eventually could be more expensive and time-consuming, for example, the preparation of reagents and fluoride standards would be required for every set of samples immediately after collection, compared to being able to store samples and perform fluoride analysis in bulk.

Plasma, on the other hand, requires preparation i.e. centrifugation, which is time consuming and requires appropriate equipment / resources, however, it can easily be stored at -18ºC for analysis and future work. Therefore, venous plasma was deemed as the most appropriate method of blood collection for studies on fluoride metabolism in humans. Venous plasma was then used as a measure of comparison with saliva collection in Study 2B.

In Study 2B, two methods of saliva collection were investigated and both methods of saliva collection were considered practical and suitable volumes were gained for fluoride analysis. However, the collection of SL-SM saliva was considered as a more reliable indicator of fluoride absorption due to the similarity of fluoride levels with literature, as oppose to whole saliva which is more prone to contamination of fluoride from other sources.

These findings will provide researchers and practitioners in the dental sector with economic and non-invasive techniques to obtain saliva samples not just for fluoride measurements, but also for biomarkers of other diseases and research in other fields.

Of all three measures used in Study 2B (whole saliva, SL-SM saliva and plasma), plasma was once again considered as the most reliable measure of fluoride absorption levels for studies on fluoride metabolism in humans. This was due to greater control of the collection method and minimal contamination of the sample, as oppose to both saliva collections. It was also the most preferred method of collection reported by participants.

As results from Study 2 (Chapter 5), indicated plasma to be the most practical method to measure fluoride absorption, this method was used as a measure of fluoride absorption in Study 3 (Chapter 6) to investigate the effects of exercise on fluoride metabolism in adults.

Initial findings from the preliminary investigation into the effects of exercise on fluoride metabolism in adults found moderate exercise to increase plasma fluoride levels and lead to a delay in urinary fluoride excretion. These findings have helped to build an understanding of the impact of exercise on fluoride absorption and excretion allowing an estimation of retention in the body.

Whilst considering that school children during their mid-morning break i.e. play time, mostly engage in physical activity of moderate-vigorous intensity (>50% of playtime duration) (Ridgers *et al.,* 2011), receiving fluoridated milk before playtime may increase the retention of fluoride in children, increasing the risk of dental fluorosis. Findings from this study provide an evidence base for stakeholders and decision makers in dental public health who may wish to review the time of fluoride supplementation schemes in schools such as fluoridated milk provision and supervised fluoridated tooth brushing scheme.

Other health professionals may also wish to review the fluoride dose and time of administration prescribed to patients such as dentists that may prescribe fluoride supplements or a highly fluoridated toothpaste to caries prone children (or even adults). This would avoid increased retention of systemic ingestion, however, would still allow the topical caries preventative effects. Although, a larger trial is required to determine the effects found, preliminary findings have provided a basis for further investigation in both, adults and children.

Findings from Study 3 and practicality of the study protocol led to the development of a practical and ethically approved protocol to investigate the effects of exercise on fluoride metabolism in children (Study 4 – Chapter 7). Ethical approval was gained and a pilot of Study 4 (Chapter 7) was undertaken. Due to challenges with recruitment and lack of time, only one participant (parent and child) was recruited. Yet, the study along with urine sample collection was undertaken with ease. Experience from the pilot study led to suggestions of slight modifications to the structure of exercise activity involved. Further work is required to determine the effects of exercise on fluoride metabolism in children, as well as adults. The overall links between the study findings in this project are illustrated by Figure 8.1.

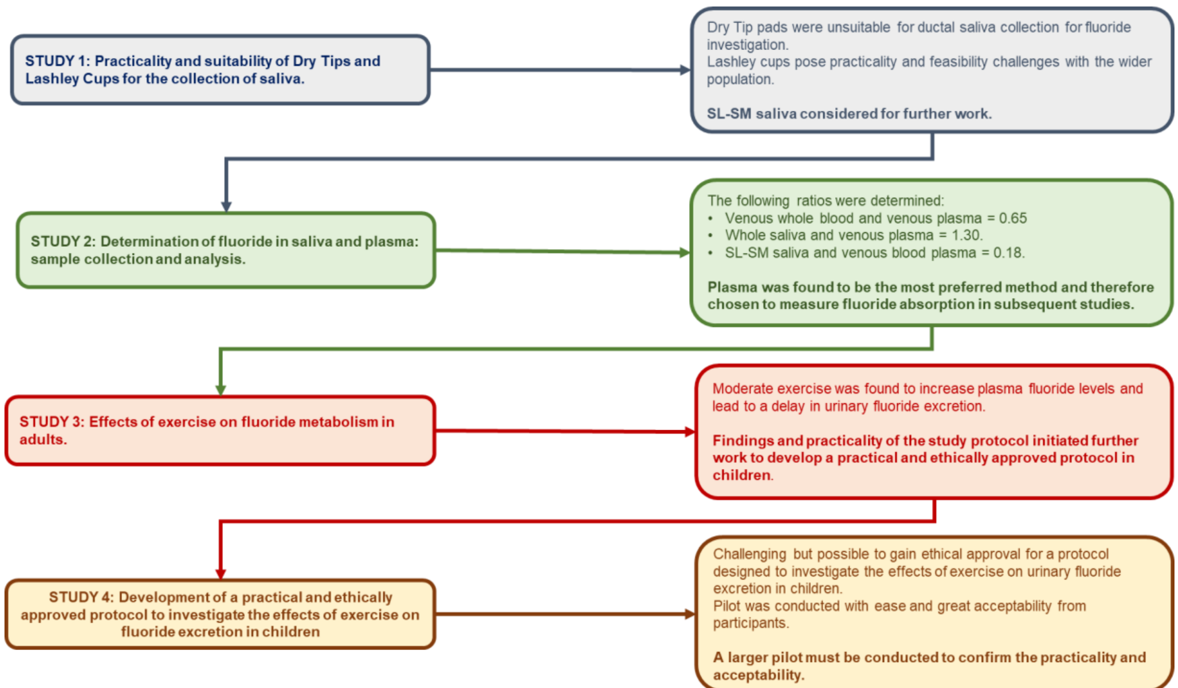


**Figure 8.1. Links between the study findings in this project.**

## 8.5. Overall conclusion and novelty of the work

This project found several conclusions from the four studies which were undertaken to gain a better understanding of fluoride metabolic responses in humans. Below are the main conclusions and novelties from each study:

**Study 1:**

- Dry Tip pads have high endogenous fluoride content and are unsuitable for the use of ductal saliva collection for fluoride investigation.
- Lashley cups require trained dental staff to collect ductal saliva. However, may still pose challenges in terms of practicality and feasibility with the wider population including children.

**Study 2:**

- Venous whole blood and venous plasma have a mean ratio of 0.65.
- Whole saliva and venous plasma have a mean ratio of 1.30.
- SL-SM saliva and venous blood plasma have a mean ratio of 0.18.
- Venous plasma is considered as the most preferred measure of fluoride absorption levels for studies on fluoride metabolism in humans.

**Study 3:**

- Moderate exercise can increase plasma fluoride levels.
- Moderate exercise can also lead to a delay in peak urinary fluoride excretion (UFE) and rate of UFE.
- The increased plasma and reduced excretion following moderate exercise suggest an increased retention of fluoride in the body.
- Vigorous exercise can lead to a delay in time to reach maximum plasma fluoride concentration.

**Study 4:**

- Although challenging, it is possible to gain ethical approval for a protocol designed to investigate the effects of exercise on urinary fluoride excretion in children.
- The pilot study was conducted with ease and great acceptability from both, the parent and child participant.

## 8.6. Future recommendations and contributions to knowledge

Overall, the below recommendations for future work and contributions to knowledge were made based on findings from this project:

**Collection of blood and saliva**

Findings from this study recommend oral health researchers to further develop the use of Lashley cups for ductal saliva collection. The design of the apparatus should be reconsidered to be able to place the cup over the parotid duct with ease, as well as alternative methods of suction to extract the saliva. The collection of SL-SM saliva using oral syringes should be performed with a larger population size with more controls in place to further assess practicality of the method and fluoride levels of SL-SM saliva.

Other devices such as the Lashley cup should also be investigated in the collection of SL-SM saliva. Further research is recommended to compare the collection and fluoride concentrations of parotid ductal saliva with SL-SM saliva. It is recommended that staff and students from the dental sector should be adequately trained to identify salivary glands / ducts and extract their respective saliva's using collection methods / collection devices developed by researchers e.g. Lashley cup. Development of a universal device which can be used to collect various types of saliva will be more cost effective and make it easier and more practical to be incorporated within the mandatory training of dental professionals.

With respect to blood collection, it is recommended to investigate dried blood spot (DBS) sampling for fluoride analysis. It is recommended to evaluate the practicality and acceptability of DBS sampling amongst adults and children. Further research can be conducted to qualitatively and quantitively test participant preference on the collection methods of saliva and blood, e.g. questionnaires before and after sample collection, etc. This will help determine the most practical and accepted methods of blood and saliva

collection, not just for studies in fluoride pharmacokinetics but within wider research and clinical settings.

**Effect of exercise on fluoride metabolism**

Findings from the adult study which have shown moderate exercise to affect fluoride metabolism provide an evidence base for recommendations for public health policy makers and clinicians in the field of oral health.

Urinary fluoride excretion (UFE) is an appropriate, practical and acceptable marker for fluoride excretion. Therefore, it is recommended that UFE should be used for the regular monitoring of fluoride intake, especially in fluoridation schemes involving young children e.g. school milk fluoridation scheme and tooth brushing scheme. This would allow to ensure the safety and effectiveness of such fluoridation schemes and regular monitoring and reviewing of the fluoride dose administered. For example, if reduced urinary fluoride excretion levels are witnessed in children following implementation of the fluoridation schemes, it would suggest an increased retention of fluoride in the body, and vice versa.

With respect to the UK school milk fluoridation scheme, it may be recommended that physical education (PE) classes should be scheduled during the morning for the age groups participating in school milk fluoridation schemes. It may also be recommended that children consume fluoridated milk after lunchtime, whereby they participate in classroom-based activities for the next 3 hours till home time. Plasma fluoride levels return to baseline levels within 3-6 hours post ingestion (Agali and Shintre, 2016), therefore any physical activity performed after school hours is less likely to affect the metabolism of fluoridated milk consumed after lunchtime.

The same recommendation would apply for the school tooth brushing scheme which involves young children participating in supervised toothbrushing using a fluoridated toothpaste. Although, the caries preventative effects of fluoride are mainly topical, unintentional ingestion of toothpaste by children can still increase their risk of dental and skeletal fluorosis. Therefore, it may be recommended that children undertake their supervised toothbrushing after lunch time as well to avoid increased retention of fluoride.

Recommendations can also be made for clinicians in practice. For example, dentists prescribing fluoride supplements or a highly fluoridated toothpaste to caries prone children

(or even adults) may recommend patients to avoid any physical activity of moderate intensity for at least 3 hours following use of fluoridated products.

Recommendations for research would involve conducting a larger investigation with adults to confirm the effects of exercise on fluoride metabolism in the wider population. The current protocol of ‘different intensities of continuous exercise’ can be repeated as well investigating the effects of ‘acute vs chronic’, ‘continuous vs intermittent’ exercise and ‘active vs sedentary’ participants. Whilst considering the importance of the physiological responses of lactate and blood pH following exercise, future studies should also try to measure lactate or blood pH to look into the association of lactate concentration and fluoride absorption.

With respect to children, findings from this project show that a protocol investigating the effects of exercise on fluoride metabolism can be conducted with ease and great acceptability by both child and parent participants.  Therefore, it is recommended that a larger pilot must be conducted to confirm the practicality and acceptability of the protocol. The finalised protocol involving ‘semi-structured physical activity’ can then be performed in laboratory and field settings. In a field setting, the protocol can be performed in primary schools with existing tooth brushing and fluoridated milk schemes.

Other structures of physical activity can also be investigated in children such as ‘different intensities’, ‘structured vs semi-structured’, ‘free-play vs structured’, ‘continuous vs intermittent’, ‘acute vs chronic’ activity and ‘active vs sedentary’ children. The various structures of physical activity can be compared in laboratory and field settings. Subsequent findings will help to further recommend time of fluoride administration in school fluoridation schemes.

It is recommended to include the effects of exercise on fluoride metabolism in the educational curriculum of students and clinicians from backgrounds of dentistry, nutrition, toxicology, biology, public health, physical education, and sports science.

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