Chapter 3

Arecoline Levels in Saliva
3.1. Introduction – Arecoline levels in saliva

3.1.1. The cellular response to arecoline

Chemical analysis of areca nut extracts reveal a range of compounds including: arecoline, guvacoline, methyl and ethyl esters of nicotinic acid, arecaidine, arecolidine and guracine (Lord et al., 2002). Amongst these, the alkaloid arecoline is particularly implicated by its genotoxic, mutagenic and carcinogenic effects in the pathogenesis of oral mucosal disease (Chang et al., 1998; Jeng et al., 2001; Lee et al., 2006).

The specific effects of arecoline on cells, in vitro, differ dependent on the cell type involved, the concentration of arecoline and the exposure time. For example, arecoline can be stimulatory (Chang et al., 2004) and cytotoxic (Chang et al., 2004; Jeng et al., 1999b; Jeng et al., 2003; Jeng JH, 1999) for keratinocytes, depending on the concentration, while fibroblasts and endothelial cells respond in a similar manner although generally at a lower concentration (Chang et al., 1998; Chang et al., 2001b; Chang et al., 2001c; Chang et al., 2001d; Harvey et al., 1986; Jeng et al., 1994; Jeng et al., 1999c; Meghji and Harris, 1995; Scutt et al., 1987; Tsai et al., 1997; Williams et al., 1998). Arecoline is also associated with increased apoptosis (Chang et al., 2004; Jeng et al., 2003).

Exposure to arecoline can induce cell cycle arrest at the G2 / M boundary in both fibroblasts and keratinocytes (Chang et al., 2001a), but did not appear to affect apoptosis in this particular study. It also depresses DNA synthesis in both keratinocytes and fibroblasts (Brandwein-Gensler and Hille, 2003; Chang et al.,...
2001a; Chang et al., 1998; Chen et al., 1995; Chung et al., 1994; Jeng et al., 1999a; Jeng et al., 1994; Jeng et al., 1999b; Nair et al., 1992; Sharan and Wary, 1992; Spalding et al., 1995; Sundqvist et al., 1989; Sundqvist et al., 1991; Wary and Sharan, 1988; Wary and Sharan, 1991; Yang et al., 2004) The depressed DNA synthesis results in reduced cell survival and lower rates of protein and DNA synthesis in a dose-dependent manner commencing at a concentration of 15μg/ml arecoline (0.1 mM) in keratinocytes (Jeng et al., 1999b).

Separately, one group reported arecoline as stimulating collagen production by fibroblasts (Harvey et al., 1986; Meghji and Harris, 1995; Scutt et al., 1987), while others have observed the reverse effect (Brandwein-Gensler and Hille, 2003; Haque et al., 2001; Shieh et al., 2004), with reports of complete inhibition of collagen synthesis (Brandwein-Gensler and Hille, 2003; Chang et al., 1999; Haque et al., 2001; Shieh et al., 2004; van Wyk et al., 1995).

Similarly, cytokine production has been reported as either reduced (Brandwein-Gensler and Hille, 2003; Haque et al., 2001) or increased in different studies (Brandwein-Gensler and Hille, 2003; Haque et al., 2001; Hsu et al., 2001). A generalized increase in a range of inflammatory cytokines has been reported in both the epithelium and lamina propria in response to arecoline, with these mediators including: interleukin (IL)-1 alpha, IL- beta, IL-6, transforming growth factor (TGF)-beta, platelet-derived growth factor, and basic fibroblast growth factor, while a reduction in interferon-gamma is seen (Haque et al., 1998). In other work, IL-8 secretion by keratinocytes has been shown to increase in response to arecoline (Cheng et al., 2000). Also IL-6 expression is significantly upregulated by arecoline and areca
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quid products (Tsai et al., 2004). Hue (2001) noted a reduction in IL-2, TNF-alpha, and TGF-beta in mononuclear circulating cells of healthy people who did not report a history of areca nut use and who were stimulated with arecoline (Hsu et al., 2001). Considering the response of fibroblasts independently, as described across multiple reports, there is a narrow range of arecoline concentrations at which cell proliferation and collagen synthesis are stimulated, this range being from 0.1 to 10 μg/ml (Canniff et al., 1986; Harvey et al., 1986; Meghji and Harris, 1995; Tsai et al., 1997). At higher concentrations, arecoline appears to be cytotoxic for fibroblasts (Chang et al., 1998; Chang et al., 2001b; Jeng et al., 1994; Jeng et al., 1999c; Tsai et al., 1997).

Similar results are reported for the effect of arecoline on keratinocytes, although these cells appear more resistant to the cytotoxic effects of arecoline. Cytotoxicity is again observed, although at higher concentrations commencing above 31 μg/ml, and causing a reduction in cell number in the order of 38% (Jeng et al., 1999c). Associated with this cytotoxicity, is an observed increase in prostaglandin E2 (PGE2) production and reduced IL-6 release by the keratinocytes (Jeng et al., 1999c).

3.1.2. Oral arecoline concentrations

The response of cultured cells to arecoline, as outlined above (3.1.1.), is highly dependent on concentration and suggests that a knowledge of arecoline concentrations in the oral cavity achieved during chewing is important to understand oral disease in nut chewers. At the time that work described in this thesis was performed, there was only one publication describing oral arecoline levels associated with areca nut chewing (Nair et al., 1985). Work described in this Chapter expands significantly upon this earlier report.
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Nair et al. (1985) used gas chromatography to quantitate oral arecoline levels in the saliva of six subjects chewing areca nut with and without tobacco. The concentrations of arecoline found in saliva varied greatly amongst subjects. It ranged from 2.4 to 142.9 µg/ml, with a mean value of 51.9 µg/ml in subjects who included tobacco with the ‘chew’. However, if tobacco was excluded, then the levels of arecoline in saliva varied from levels below the sensitivity of the detection system up to 89.9 µg/ml, with a mean value of 29.7 µg/ml. Six subjects were included in each of the two groups. Unfortunately, it was unclear as to whether the lower arecoline levels associated with the use of tobacco reflected some particular effect of the tobacco, or of chewing habits associated with the presence of tobacco, or some other factor.

The high arecoline concentrations reported by Nair et al. (1985) in some individuals, would appear to be sufficient to stimulate collagen synthesis and also to reduce fibroblast proliferation, while there may also be keratinocyte cytotoxicity (Jeng et al., 1999b; Jeng et al., 2001; Wary and Sharan, 1991). Interestingly, however, the subjects in Nair’s study were not reported as having clinical signs of oral submucous fibrosis or any other habit-associated lesions. This is consistent with the notion that the period of exposure is important, as well as perhaps individual, and potentially idiosyncratic, responses to areca nut. It is further possible that although substantial cellular injury occurs in many individuals using areca nut, that there is often no apparent detectable clinical change, with the consequence that epidemiological surveys for detectable oral disease may not fully assess the impact of areca nut use. This idea is consistent with the reported increased incidence of oral squamous cell carcinoma (OSCC) amongst areca nut users, despite relatively modest detectable premalignant changes (Merchant et al., 2000). With regard to this, it is important to note
that increased keratinocyte ‘turn-over’ would be expected in response to arecoline cytotoxicity, and that such accelerated cellular turn-over is consistent with an increased incidence of malignant change.

3.1.2.i. Likely penetration of arecoline across the mucosal barrier

While acknowledging the concentration dependent effects of arecoline upon fibroblasts and keratinocytes outlined above in 3.1.2, it is important to recognize that this can only have biological meaning if arecoline is able to penetrate the mucosal barriers to enter the tissues. The potential of arecoline to enter the tissues and hence the circulation via the oral epithelium is important to properly interpret data presented in the chapter, and for this reason it is necessary to first discuss literature relevant to this issue.

By way of observation, the streets and thoroughfares of communities that indulge in areca nut chewing frequently have coloured markings on the ground where chewers have expectorated, discarding the areca nut along the path. This habit seems extremely common amongst users of the areca nut, and most users do not consciously swallow either the saliva or the nut. Thus while it is impossible to say that the effects of chewing the areca nut are obtained without swallowing it definitely seems to be the custom that the majority of users expectorate most or all of the saliva containing the areca nut and arecoline. Therefore, assuming that arecoline is a significant psychoactive ingredient, then appreciable levels of circulating arecoline are likely achieved by means of absorption across the oral mucosa prior to expectoration for most users.
3.1.2.ii. Demonstrated permeability of the oral mucosa

One of the primary functions of the oral mucosa is to provide a barrier, protecting the underlying tissue from substances in the oral cavity, as well as preventing the loss of body fluids into the oral cavity. This barrier function is not complete and many substances have been shown to pass across the epithelial surface (Siegel, 1984). This fact is utilized for administration of a number of medications including glycerol trinitrate, neostigmine, hyoscyamine, buprenorphine and buprenorphine/naloxone, all of which require sublingual administration (Marsh and Marsh, 2000; Moffat, 1971). These drugs not only penetrate the mucosa, but clearly achieve circulating concentrations sufficient for systemic therapeutic effects. In view of the psychotropic effects observed with areca nut chewing, as well as the known identical effect of arecoline (Asthana et al., 1996), it seems likely that similar pharmacokinetic effects are at work with arecoline during areca nut chewing.

The thickness and structure of mucosal surfaces greatly affects permeability. Mucosae with a recognized role in absorption such as those of the gastro-intestinal tract, tend to have a single layered epithelium. The oral mucosa, however, is covered by stratified squamous epithelium, while three different types of this epithelium are seen, each reflecting the highly localized functional demands of different parts of the oral cavity. The 'masticatory' mucosa covers the gingiva, hard palate and other areas subjected to significant mechanical forces of mastication. It has a keratinised epithelium and represents approximately 25% of the surface area in the oral cavity (Collins and Dawes, 1987; Squier, 1991). Approximately 60% of the oral cavity is covered by a non-keratinized 'lining' mucosa, which provides an elastic, deformable surface capable of stretching with movement during mastication and speech (Collins and
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Dawes, 1987; Squier, 1991). Being both non-keratinized and relatively thin, this area is the most permeable to exogenous agents. Finally, the ‘specialized’ mucosa covers the dorsum of the tongue which represents approximately 15% of the oral mucosal surface, and has properties in some ways intermediate to both the lining and masticatory mucosae, with both keratinised and non-keratinised epithelium (Collins and Dawes, 1987; Squier, 1991).

The main sites for absorption of drugs across the oral mucosa are the buccal and sublingual areas, in which the lining mucosa is very thin and richly vascularized. The oral mucosa is approximately ten times more permeable to water than skin. While there are differences in permeability to water within the mouth, these are relatively minor. For example, there is only a two-fold difference between the least permeable mucosal surface, being the palate, and the most permeable surface, which is the floor of the mouth. The buccal mucosa and lateral border of the tongue have a permeability value intermediate between that of the palate and floor of the mouth (Lesch et al., 1989).

3.1.2.iii. Mechanisms of transport across the oral mucosa

Substances can cross various epithelial membranes by means of simple diffusion, carrier-mediated diffusion, active transport or pinocytosis. However it appears that within the oral cavity the main mechanism is simple diffusion, and to a lesser degree carrier-mediated transport (Siegel, 1984).
3.1.2.iv. Transport by simple diffusion

Simple diffusion across an epithelial barrier can be mathematically modelled and has been expressed in the following equation (Siegel, 1984):

\[ K_p = \frac{Q}{A.t.(C_0 - C_i)} \]

Where:

- \( Q \) is the quantity of compound traversing the tissue in time ‘\( t \)’ (minutes)
- \( C_0 \) and \( C_i \) are the concentration on the outer (epithelial) and inner (lamina propria) sides of the specimen
- \( A \) is the area of exposed tissue, measured in square centimetres - \( \text{cm}^2 \)
- \( K_p \) is the permeability constant, and is expressed in units of centimetres per minute.

3.1.2.v. Solute properties influencing permeability

3.1.2.v.1. The oil/water distribution ratio (R)

Comparison has been made of the oil : water ratios (R values) of several alkaloid drugs with the ratios of sublingual to subcutaneous doses needed to produce similar pharmacological effects (Walton, 1935). Drugs with a high values for R require much lower doses than drugs with lower R values in order to produce pharmacological effects similar to those obtained by subcutaneous injection (Siegel, 1984). The effect of this is that the more lipophilic an agent is, the more effectively it will penetrate the epithelial barrier.
3.1.2.v.2. The effect of concentration gradient on diffusion rate

Consistent with the equation shown above, is a linear relationship between solute concentration gradient and the amount transferred across a diffusion barrier. For example, a 10-fold increase in the concentrations of either of the local anaesthetic agents lignocaine or prilocaine, results in an approximate 10-fold increase in the rate of transfer across canine lingual mucosa (Bergman et al., 1969), while a similar relationship is reported for ascorbic acid (Alvares and Siegel, 1981). Linearity of absorption has also been demonstrated over a 32-fold range of concentrations for two ‘adrenergic’ blocking drugs, propanolol and antenolol. Although these observations support the importance of simple diffusion in the oral absorption of drugs, a possible additional contribution by facilitated diffusion can be neither excluded nor readily confirmed from such limited kinetic data (Siegel, 1984).

3.1.2.vi. The effect of pH and other environmental factors on mucosal absorption

Observations suggesting that acidic and basic drugs penetrate the oral mucosa by passive and not facilitated diffusion, include identical absorption from mixtures as from purified preparations, and an identical percentage absorption regardless of concentration. Important for the current study, is that absorption of agents which are either weak acids or bases, depends critically upon the relative concentrations of non-ionised material, rather than the total concentration of substance present. It is also important to note that pH has a profound effect on the charge state of drugs, and thus has the potential to significantly affect passage across the oral mucosa.
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Most drugs are weak organic electrolytes and the extent of ionisation is important in determining rates of membrane penetration (Moffat, 1971). Increasing the pH of the oral environment will facilitate the absorption of basic drugs, since the concentrations of lipid soluble non-ionised forms increases, while a similar effect is seen for acidic drugs when the pH is reduced. While the pH of saliva lies between 6.0 and 7.4, changes in salivary pH affect the proportions of weak organic acid or base in non-ionised forms, and thus the rates at which such compounds cross the oral mucosa.

Under physiological conditions, the absorption of weak electrolytes with pKa values close to physiological pH are significantly affected by slight changes in oral pH (Siegel, 1984).

When considering arecoline, which has a pKa of 6.84, there is a preponderance of non-ionised, and thus lipophilic molecular forms in saliva. If an alkaline environment is created in the oral cavity, by including slaked lime with the betel quid, there is ready hydrolysis of uncharged arecoline to the negatively charged arecaidine (Von Nieschulz, 1968). Arecaidine penetrates the mucosa at a slower rate than arecoline, which is its methyl ester, while conversely, neutral or acidic conditions appear to promote transmucosal diffusion of arecoline (van der Bijl et al., 2001).

Separately, mucosal permeability can also be altered independent of pH by exogenous substances, including surface-active agents widely used in toothpaste and mouthwashes (Siegel, 1984). Some enzymes and mucolytic substances are also reported to enhance penetration of the oral mucosa, presumably by removing or altering surface coverings. The presence of areca nut extract itself, leads to a
reduction in permeability in the order of 20% as compared with controls (van der Bijl and Thompson, 1998).

3.1.3. Oral mucosal permeability can be changed by systemic factors

3.1.3.i. The effect of nutritional deficiencies on mucosal absorption

Systemic factors can alter the permeability of the oral mucosal membrane. Measurements of permeability, *in vitro*, demonstrate that vitamin C deficiency leads to an increase in mucosal permeability (Alfano *et al.*, 1975; Alvares and Siegel, 1981; Siegel, 1984). In addition, the permeability of the sulcular epithelium is decreased in patients given supplements of vitamin C (Siegel, 1984). From this it is perhaps not surprising that malnutrition, also associated with alcohol abuse, is linked to discernable changes in mucosal integrity (Enwonwu, 1994; Enwonwu and Meeks, 1995; Strickland, 1998).

3.1.3.ii. The effect of age and oral mucosal disease upon absorption

Clinically mucosal atrophy is observed with age; however, there seems no clear effect of this apparent thinning on mucosal permeability (Alfano, 1978; Squier, 1991).

Separate to ageing, is oral mucosal atrophy and necrosis associated with pathological conditions such as lichen planus, viral infections, pemphigus, allergic reactions and
Radiation or chemotherapy-associated mucositis. In such situations, atrophy or loss of the epithelium, with or without altered mucin production, may disrupt the major permeability barrier (Squier, 1991).

3.1.4. A need for further investigation of oral arecoline concentrations and mucosal absorption

Although a wide range of intraoral arecoline concentrations are reported consequent to chewing the areca nut, with or without tobacco, a need for further evaluation is apparent (Nair et al., 1985). Unfortunately, the published study does not provide information on the kinetics of oral arecoline levels during chewing, or any potential daily variability in concentration.

In addition, the basis for the wide range of concentrations observed remains unclear from the single available published study (Nair et al., 1985). Salivary concentrations are reported to range from 2.4 to 142.9 μg/ml when tobacco is included in the ‘chew’, and from 0.0 to 89.9 μg/ml when tobacco is not included, with corresponding differences in mean arecoline level between the two groups. Assuming that Nair et al. (1985) provided a similar amount of areca nut to each participant, the highly variable salivary concentrations observed suggest potentially interesting kinetic or other idiosyncratic variability amongst users of the nut, as well as possible effects of tobacco on the release, uptake and distribution of arecoline between saliva and tissues.
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As noted in Chapter 2, oral mucosal changes are present in a sub-group of the population using the areca nut. The frequency of such presentations is reported to reach up to 40% of the areca nut chewing population (Pearson et al., 2001), and although this is substantially higher than observed in the study described in Chapter 2, the wide range in the incidence of oral lesions seen could reflect underlying differences amongst users in the concentrations of salivary arecoline achieved during chewing.

3.1.5. A need for development of a new protocol for the measurement of salivary arecoline using high pressure liquid chromatography

Most of the burden of disease related to the use of the areca nut is within the developing countries of Asia, so that research into areca nut use is facilitated by development of relevant laboratory techniques that may be more readily available in these countries. Previously reported studies used gas chromatography to determine arecoline concentration or to distinguish arecoline from other alkaloids in saliva (Nair et al., 1985; Self et al., 1999). One objective of the study described in this Chapter was to develop an alternative approach to arecoline measurement which was of comparable accuracy to gas chromatography but more accessible. As described in this Chapter, high performance liquid chromatography (HPLC) was found to be an effective alternative technology to gas chromatography, while at the same time being more widely available in the developing world.
3.2. Materials and Methods

3.2.1. Materials

3.2.1.i. High pressure liquid chromatography and mass spectrometry equipment

A Shimadzu HPLC-MS system was used for the detection of arecoline (Shimadzu Pty Ltd Kyoto, Japan). The HPLC system included a LC 10AD model dual piston pump, DGU-3A degasser, SIL-10A automated injector, CBM-10A controller, and a CTO-10A oven. All experiments used a Luna 5 μm particle size, 100 Å pore size, 250 mm x 4.6 mm internal diameter, stainless steel, C18 reversed-phase column (Waters Pty. Ltd., Sydney, Australia). Electron spray mass spectrometry (MS) was used to detect arecoline (Shimadzu Pty Ltd, Kyoto, Japan), with an atmospheric pressure chemical ionisation electro-spray interface (ESI), controlled by Shimadzu HPLC (Version 1.0) software on a 486 PC computer.

3.2.1.ii. Reagents

Arecoline (methyl-1,2,5,6-tetrahydro-1-methylnicotinate), arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid), triethylamine, and caffeine were from Sigma Pty. Ltd. (St Louis, USA). HPLC analytical grade acetonitrile was from Unichrom Co (Kuang-Fu Rd, Taipei 100, Taiwan ROC). Lignocaine was from Astra Zenica (North Ryde, Sydney, Australia). Areca nut was obtained from local asian ‘nut and spice’ shops in Sydney.
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The areca nut chips were obtained supplied in 50 gm packages from Roshan Naidu’s (Importer and Distributor, Parramatta, NSW), while the inert rubber-based impression material was 3M Imprint II (vinyl polysiloxane, Light body) from 3M Dental Products, (St Paul MN).

3.2.1.iii. Plastic ware

Ten mL polyethylene tubes with screw tops as well as Eppendorf disposable plastic tubes were purchased from Crown Scientific Pty. Ltd (Sydney Australia). Disposable C₁₈ extraction cartridges were from Alltech (Deerfield, IL., USA).

3.2.1.iv. Calibration solutions

Calibration solutions of arecoline from 50 to 500 ng/ml, and lignocaine from 0.001 ug/ml to 50 ug/ml were prepared in Milli-Q water via serial dilutions.

3.2.2. Methods

3.2.2.i. Questionnaire completed by volunteers

To obtain relevant information about participants, a survey essentially similar to that used for the earlier Australian survey (Appendix) was used. Important refinements included questions relating to the different localities of habitation, the type of product used, the frequency of use, the amount chewed, the duration of chewing and the last occasion when the habit was practised.

The modified questionnaire was reviewed from the perspective of clarity of meaning and flow of questions by two staff members at the Westmead Centre for Oral Health
as well as by four students, three of whom spoke a language other than English at home. Minor modifications were made in response to this review. An important change was inclusion of a word list in multiple languages for the areca nut, which recognized that while some participants may be unfamiliar with the term ‘areca nut’, they would be familiar with the traditional name of the nut.

3.2.2.ii. Collection of mixed and parotid saliva

Thirty-two subjects who had an areca nut chewing habit were recruited into the study. Mixed saliva was collected from 32 subjects who were asked to spit into 10 ml disposable centrifuge tubes before being given 0.5 gm of areca nut to chew, and then collecting all saliva generated by spitting into further centrifuge tubes, with fresh tubes provided at 1, 3, 5, 10, 15, 20 and 25 minutes. Participants were then asked to remove all remaining nut particles from the mouth, and then saliva samples were collected for up to a further 20 minutes, changing tubes at five minute intervals.

While thirty-two subjects provided saliva during areca nut chewing, six of these subjects repeated the procedure over three consecutive days. In addition, parotid saliva was also collected from these six subjects using a Lashley cup, normally placed over the punctum of the right parotid duct.

Saliva samples were centrifuged at 402 x g (times gravity or 3,000 rpm), decanted into fresh tubes and snap frozen in liquid nitrogen before storage at -20°C. Controls consisted of subjects with no reported areca nut chewing habit, who instead of being given areca nut to chew, were asked to chew inert rubber-base impression material cut to approximate a size similar to the 0.5gm of areca nut.
3.2.2.iii. Determination of salivary flow rates

Salivary flow rates were determined by weighing centrifuge tubes and assuming a specific gravity for saliva of 1g/ml. Parotid salivary flow rates were determined both before and after chewing areca nut. In brief, saliva was collected using Lashley cups placed over the opening of Stenson’s duct. Stimulation of salivary flow was achieved by giving the subject 5.0 gm of areca nut to chew. The volume of saliva was measured in tubes by weight assuming the specific density of saliva to be 1g/ml. The flow rate for each subject was expressed in ml/min. All samples were collected in the morning between 9 am and 11.30 am. Mixed saliva was also collected from these individuals by asking subjects to spit into 10 ml plastic cups. The measurements of both the mixed and parotid saliva were recorded over the three consecutive days.

3.2.2.iv. Conditions for detection of arecoline by mass spectrometry

MS instrument parameters were optimised to obtain protonated molecules together with some characteristic fragments. The following ESI-MS conditions were found to be effective for detection of arecoline: nitrogen for drying was heated to 350°C and applied at a flow rate of 10.0L/min; nitrogen as a nebulizer was applied at a pressure of 40 psi; capillary voltage was set at 1500V; fragmentor voltage applied at the exit end of the capillary was at 110V; the dwell time was 139 ms; and the mass peak width was 0.7\textsuperscript{th} (FWHM). Qualifying ions were identified with mass : charge (m/z) of 156, and 197 for arecoline, and m/z 235 for lignocaine. The acceptanice criterion for ion intensity ratios was a deviation of approximately 20% of the average of the ion intensity ratios of all the calibrators. The ions at m/z 156 and m/z 197, for arecoline,
were used for quantification. These optimum MS parameters for arecoline were determined following direct infusion of a solution in acetonitrile into the mass spectrometer. The mass spectrum obtained by negative ion electron-spray contained an intense [M-H]- molecular anion isotope group (Fig 3.1). Two of these ions, at m/z 156 and m/z 197 produced similar product-ion mass spectra when subjected to collision-induced dissociation using argon. Lignocaine with a m/z of 235 was used as the internal standard. Factors other than concentration contribute to the height and width of peaks measured in MS spectra. To correct for this variability, arecoline levels in individual samples were expressed in terms of a ratio between peak heights for the arecoline peak as compared with that of the internal lignocaine standard.

3.2.3.v. The preparation of samples for HPLC/MS

3.2.3.v.1. The preparation of saliva samples for HPLC

All saliva samples were centrifuged to remove areca nut and any solid material prior to freezing. Fifty-micro-litre volumes of thawed saliva were mixed with 50μl volumes of stock Lignocaine and added to 200 μl of acetonitrile. In most cases, the concentration of stock lignocaine was 3 μg/ml, however, where arecoline levels were very low and required re-evaluation by HPLC, the stock solution of lignocaine was at a concentration of 1.5 μg/ml. Solutions were vortexed for 1 minute and centrifuged for 5 minutes prior to HPLC.
Figure 3.1

A typical mass spectrograph for arecoline dissolved in water. Major peaks for arecoline was at m/z 156, (and 197 for an adduct of arecoline) and lignocaine was at 235, and these were used for quantitation. Minor peaks were also observed.
3.2.3.v.2. The preparation of standards for quantitation of arecoline in saliva

To prepare standards for quantitation of arecoline in saliva, fresh saliva samples from the author, were spiked with arecoline at increasing concentrations to achieve final concentrations in saliva ranging from 0.01 µg/ml to 50 µg/ml. In addition, lignocaine (3µg/ml) was used as an internal standard. The ratio of the relative proportion of arecoline to lignocaine, as determined by the height of the MS spectral peaks was used to establish standard curves ranging in arecoline concentration from 0.01 to 50 µg/ml. To further evaluate the accuracy and reproducibility of this measurement approach, fresh saliva samples were spiked with 50 ng/ml of arecoline and repeatedly measured. Recovery was estimated by using four different concentrations of arecoline in duplicate spiked saliva: 100, 150, 200, 250, and 300 ng/ml.

3.2.3.v.3. The effect of pH on the recovery of arecoline from HPLC/MS

To determine the effect of pH on arecoline recovery, aliquots of Milli-Q water were adjusted with a solution of 0.1 M sodium phosphate and a solution of 0.1M calcium carbonate to achieve pH values of 4, 6, 7, 10.5 and 11, with the pH level confirmed using a TPS digital pH meter (Model 1852, TPS Pty Ltd Brisbane). The solutions were then spiked with arecoline, 1 µg/ml, prior to the evaluation of arecoline concentration by MS.
3.2.3.vi. High pressure liquid chromatography

Samples of neat saliva were analysed by reverse-phase chromatography using a Luna C\(_{18}\) column at 30°C and a mobile phase composed of 50% acetonitrile and 50% 0.01 M sodium hydrogen-phosphate (pH 7.8) with 0.01% triethylamine at isocratic flow rates ranging from 0.3 to 1.2 ml/min. Dependent on the levels of arecoline found in samples, accurate quantitation with reference to standards required application of either 3, 6 or 10 ul volumes to the column. Where the concentration of arecoline was less than 1 µg/ml, samples were diluted at a ratio of 1:2 with Milli-Q water in acetonitrile.

3.3. Results

3.3.1. Salivary arecoline measurements by mass spectrometry were reliable and reproducible

As indicated in Figure 3.1, arecoline appeared as a discrete spectral peak with MM of 155 Da, and a column retention time of 1.6 min, while the internal standard found most appropriate for MS detection was lignocaine, with a MM of 234 Da and a column retention time of 3.7 min. It was possible to quantitate arecoline concentration using MS spectra, by measurement of the peak height specific for arecoline, and comparison with a standard constructed from a range of samples of known concentration (Figure 3.2). Standards were assessed each time that MS was performed, so that new standard curves were prepared for each experimental session. The lower limit of detection was 50 pg of arecoline.
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When further characterizing the reproducibility of arecoline measurement in this way using saliva spiked with arecoline (50 ng/ml) and repeating measurements 5 times on one occasion, there was less than 1.2% variability in the recorded measurements. Providing further confidence in the reproducibility of arecoline measurements by HPLC / MS, when single samples were assessed on 5 separate occasions, there was at most only 2.5% variability between measurements. Importantly, when individual samples were evaluated for arecoline concentration up to 2 months apart, there was no significant or consistent variation in quantity detected, indicating sufficient stability of arecoline in saliva for meaningful quantitation of samples over time.
Figure 3.2

Arecoline standard curves for concentrations ranging from 0.01 – 1 µg/ml (A) and 0.01 – 50 µg/ml (B) relating the ratio of the peak height of arecoline to lignocaine. A linear relationship was consistently seen between the concentration of arecoline and the ratio of arecoline and the internal standard, Lignocaine. The standard working solutions for arecoline were as follows: 50µl of arecoline (1M) in water plus 50µl of 3µg/ml of Lignocaine in water plus 200µl of Acetonitrile. The mobile phase was 30%ACN, 10mM NH₄F, 0.0025%FA, pH5-6, 0.2ml/min.
A

Ratio vs. Concentration (µg/ml)

- Equation: \( y = 0.128162x \)
- \( R^2 = 0.999511 \)

B

Ratio vs. Concentration (µg/ml)

- Equation: \( y = 0.127074x \)
- \( R^2 = 0.999601 \)
3.3.2. Ionization of arecoline increased with rising pH

The concentration of arecoline in Milli-Q water, as detected by HPLC/MS, varied with the alteration of the pH of the solution. Figure 3.3 demonstrates an apparent variation in the concentration of arecoline (relative to a neutral pH of 7) in acidic and alkaline solutions. This possibly reflects increasing ionization of arecoline with increasingly alkaline conditions. It was found that arecoline measurements increased substantially with rising pH, such that at pH 10 there was approximately three times more arecoline detected while at pH 10.5 this rose to over four times the level detected at pH 7. There was a minimal arecoline detected in acidic conditions.
Figure 3.3

The concentration of arecoline as detected by HPCL/MS in aqueous solutions of varying pH. The detected concentration of arecoline altered according to the pH of the solution. High levels were detected in alkaline solutions with the highest level at pH 10.5, being over four times that at a neutral pH.

Arecoline solution of 1μg of arecoline in 20 μL of Milli-Q water (equivalent to 500ng on the column)
3.3.3. Participants were primarily from the Indian Subcontinent and used areca nut on a regular basis

Table 3.1 summarizes the data obtained from the questionnaire for participants in this study. The majority of the 32 participants who used the areca nut were from the Indian sub-continent, with 17 coming from India, 6 from Sri-Lanka, and 1 from Bangladesh. Other participants using the nut were from Malaysia (1), Taiwan (1) and Australia (6). Four of the 6 participants who did not use the areca nut but who participated as control subjects were from India, while 2 were from Sri Lanka.

Approximately one half of participants were male (53%), while the age of participants varied from 17 to 49 years, with a mean age for males of $28.8 \pm 10.5$ years and $34.2 \pm 10.1$ years for females.

The frequency of areca nut use was important for this study, since it was considered possible that arecoline levels might be constitutively high in users of the nut. The distribution of ‘frequency of use’ for the areca nut by participants according to sex is shown in Figure 3.3, and it is apparent that most participants used areca nut on a regular/semi-regular basis with 78% using it at least once a month.

The age at which areca nut use commenced is also displayed in Figure 3.4. Most participants commenced use of the areca nut during early childhood or adolescence with the earliest age of use being 5 years, although a few participants first used the areca nut in early adult life,
### Table 3.1 Summary of data obtained by questionnaire from participants

<table>
<thead>
<tr>
<th>Participant</th>
<th>Age</th>
<th>Sex</th>
<th>Ethnicity</th>
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<th>Fréquency of areca nut use</th>
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<tr>
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<td>F</td>
<td>Goan</td>
<td>7</td>
<td>1 / Month</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<tr>
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<td>39</td>
<td>M</td>
<td>Indian</td>
<td>14</td>
<td>1-2 / Month</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>F</td>
<td>Indian</td>
<td>19</td>
<td>2-3 / Month</td>
</tr>
<tr>
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<td>3 / Year</td>
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<tr>
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<td>M</td>
<td>Aust (Indian)</td>
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<td>3 / Month</td>
</tr>
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<td>25</td>
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<td>4 / Month</td>
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</tbody>
</table>

Controls

<table>
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<th>Age</th>
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<th>Ethnicity</th>
</tr>
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<td>F</td>
<td>Indian</td>
</tr>
</tbody>
</table>

Thirty two subjects indicated they had an areca nut chewing habit, while 6 subjects did not report any areca nut use and comprised the control group. There was no significant difference between males and females with regard to age, while the mean age was \(31.3 \pm 10.5\) years, with the mean ages of males (17) and females (15) being \(28.8 \pm 10.5\) and \(34.2 \pm 10.1\) years respectively. (The mean age for the control was \(36.7 \pm 9.0\) years)
Figure 3.4

Histogram demonstrating the frequency of areca nut use amongst a sample group of Australian females (closed bars), and males (open bars). Approximately 16% of the subjects used areca nut more than four times in a month, 80% of the females and 76% of males chew areca nut on a monthly basis, while only 22% considered themselves as occasional users of the areca nut.
Figure 3.5

Histogram showing the age at which participants first started to use the areca nut.

Twenty-eight percent of participants commenced using the nut before the age of 10, with 66% commencing between the ages of 10 and 20.
3.3.4. Arecoline levels in whole mixed saliva before, during and after chewing areca nut were sufficient to stimulate cellular responses and cytotoxicity

Figure 3.6 shows the maximum and minimum values for salivary arecoline observed in individual participants in each experiment. It is clear that in all cases studied the levels of arecoline in saliva of individuals chewing areca nut exceeded either the 0.1 µg/ml or the 10 µg/ml thresholds for cell stimulation or cytotoxicity respectively.

Only seven of the 32 areca nut chewers studied had no detectable arecoline in their saliva at the commencement of the study, '0' time point, so that 25/32 (78%) of subjects had residual salivary arecoline from earlier chewing episodes. Baseline levels of arecoline measured before chewing ranged from undetectable levels to 2.4 µg/ml, and in 21 of the subjects the level was above the 0.1 µg/ml, considered sufficient to stimulate cellular responses.

The maximum concentrations of arecoline achieved during areca nut chewing ranged from 5.66 to 97.39 µg/ml. No clear difference was seen between male and female subjects with regard to the peak concentrations of arecoline achieved (Figure 3.7).
Figure 3.6

The range of concentrations of arecoline detected in saliva of individual participants chewing areca nut, with participants coded as numbered in Table 3.1. In all subjects studied, arecoline levels reached concentrations above the 0.1 µg/ml reported to stimulate cell activity, while in 24 out of 32 subjects, arecoline levels were above the 10 µg/ml concentration threshold (marked with a horizontal dashed line) reported to cause cell death.
Figure 3.7

Maximum concentrations of arecoline achieved during areca nut chewing according to sex. In all cases, arecoline levels were sufficient to stimulate cellular responses (>0.1 μg/ml), or cytotoxicity (>10 μg/ml) (marked with a horizontal line), while there was no clear difference between the sexes with regard to maximum levels of arecoline achieved.
Arecoline Levels in Saliva

Peak levels of salivary arecoline were always reached during the time of chewing ($p < 0.0001$), although the precise time that this occurred varied during the 25 minutes that participants chewed areca nut (Figure 3.8), with high peak levels usually reached by 3 minutes of chewing ($p < 0.03$). In 14 participants, more than one peak in salivary arecoline concentration was observed (Figure 3.9), and this occurred before the highest peak level was reached in six cases. In one participant, there was a surprising third and lower peak in salivary arecoline levels collected between 40 and 45 minutes, being 20 minutes after the nut had been removed from the mouth. No significant difference was noted between males and females or with regard to the response of people with different countries of origin. Peak concentrations for arecoline in saliva was rarely the same between the subjects despite the same quantity of areca nut being chewed.

Although arecoline levels were substantially lower after removal of the nut from the mouth at the 25-minute time point ($p < 0.001$), levels still exceeded those required to stimulate cells in culture in most participants ($p < 0.001$) (Figure 3.8). Note that the concentration of arecoline in saliva was generally above 0.1 μg/ml.
Figure 3.8

Arecoline concentrations in saliva samples of all subjects chewing areca nut over a 25 minute period (arrow) as well as for 15 minutes after removal of areca nut from the mouth. The concentration of arecoline was above 0.1 μg/ml in all subjects for almost all of the time (dotted line), while the 1.0 μg/ml (light intermittent line) threshold was reached most of the time in most subjects and the cytotoxic concentration of 10 μg/ml (dashed line) was achieved in all but five cases.
Figure 3.9

Times at which the highest, second highest and third highest peak salivary concentrations were reached in participants. In most participants, the highest levels of arecoline were achieved within 3 minutes of chewing, while in one subject, this did not occur until shortly before removal of nut from the mouth at 25 minutes (arrow). A second and lower peak concentration was observed in many participants, while in one case, a third and still lower peak was found in the final salivary sample collected.
Number of Participants

Time (Minutes)

- Highest Peak
- Second Peak
- Third Peak
3.3.5. Secretion of arecoline by the parotid glands

To investigate the possible secretion of arecoline by the salivary glands, parotid and whole mixed saliva was collected simultaneously from six subjects. Figure 3.10 summarises the observations of experiments with all six participants. Parotid salivary arecoline levels were typically an order of magnitude lower than those found in mixed saliva ($p < 0.01$). Levels of arecoline in parotid saliva paralleled those in whole saliva, although a delay of up to 30 minutes was seen in peak parotid salivary levels for 4 out of 6 subjects. Similar results were obtained for individuals on each of the three days that they were studied, particularly with regard to the highest concentrations of salivary arecoline achieved, while the major differences between individuals were seen across all three experiments performed on consecutive days.
Figure 3.10

The concentration of arecoline (μg/ml) in parotid saliva over three consecutive days (●, ⊕, ×), as well as in whole mixed saliva during parotid saliva collection (■, △, ▲) for each of the six subjects participating in the study. Higher levels of arecoline were always detected in whole mixed saliva as compared with parotid saliva. There was a high level of consistency for readings over consecutive days for individual subjects. Two of the subjects (E,F) had arecoline levels in the parotid saliva consistently below 0.1μg/ml whereas four subjects (A,B,C,D) had concentrations that rose up to 1 ug/ml, and two of these (C,D) rose above this level, though not consistently. The presence of appreciable levels of arecoline in parotid saliva suggest secretion of arecoline in the saliva after initial absorption, and this appeared to occur within 10 minutes of initiating chewing.
3.3.6. **Areca nut chewing substantially increased salivary flow rates**

Figure 3.11 shows differences in salivary flow rate between individuals during and after areca nut chewing. The mean salivary flow while chewing the areca nut was 1.6 ml/min, (SD 0.9), while chewing, and 1.0 ml/minute (SD 0.5) after the nut was removed from the mouth (p<0.000). The flow of parotid saliva via Stensen’s duct was 0.2 ml/min while chewing the nut, and 0.1 ml/min after the nut was removed from the mouth. There was no significant difference in the flow rates of the parotid gland.
Figure 3.11

Scatter diagrams demonstrating the salivary flow rate with or without areca nut chewing in whole mouth saliva (A) and parotid gland saliva (B).

**A.** Individuals chewing the areca nut had a mean whole saliva flow of 1.6 ml/min ranging from 0.06 to 4.5 ml/min and then reduced to 1.0 ml/min ranging from 0.02 to 2.3 ml/min after chewing ceased. The difference in flow rate between chewing with and without areca nut was significant \( p<0.007 \).

**B.** Similar observations were made for parotid flow where areca nut chewing induced a mean flow rate of 0.2 ml/min, ranging from 0.01 to 0.9 ml/min and dropping to 0.1 ml/min, range 0.002 to 0.4 ml/min, after chewing. The difference in flow rate in parotid secretions between chewing with and without areca nut was again significant \( p<0.02 \).

(The flow rates were measured over varying time periods (1, 2, 5 and 10 minute intervals).)
3.4. Discussion

This is the first time that HPLC has been used to determine the concentration of arecoline in saliva. The use of HPLC allows for a relatively rapid and inexpensive method for determining the concentration of arecoline in saliva, thus making this technique available to many developing countries, which have a high prevalence of oral habits involving the areca nut. Hence this is a means of enabling basic research in countries that experience a heavy disease burden that is linked to this habit.

It was anticipated that the results obtained using reverse-phase liquid chromatography with the HPLC-MS system would be comparable to those obtained with the gas chromatography method for determining the concentration of arecoline in saliva (Nair et al., 1985), although HPLC had not been previously used in this regard. A high level of reproducibility and precision was achieved as demonstrated in experiments spiking saliva with known quantities of arecoline. The results of the present study demonstrated arecoline at a level slightly lower than that found in the study by Nair et al (1985). Nair demonstrated levels of arecoline in the saliva up to 140 μg/ml with a mean value of 52 μg/ml in subjects who both smoked cigarettes and chewed areca nut, although levels were lower (mean of 30 μg/ml) in subjects who had the single habit of chewing areca nut. The results of the present study gave a maximum arecoline concentration of 97.4 μg/ml with a mean of 37.2 μg/ml during the ‘chewing’ phase of the study. It would therefore seem that the results of the current study confirm those of Nair, although the methodology in the Nair study is perhaps less clear since saliva samples were obtained once or twice during chewing and then snap frozen and stored at -20°C. The present study found that the level of arecoline was always higher if the residue of the nut had not been removed from samples prior to
freezing. Indeed each cycle of ‘freezing-thawing’ lead to an increase in the level of arecoline concentration if remnants of the nut were not first removed from the saliva. (Nair et al., 1985).

The presence of arecoline in the saliva of some of the subjects prior to the study prompted a re-examination of the data related to the breakdown of arecoline in the body. The presence of a second peak in many subjects also raises the question as to whether arecoline is re-secreted in the saliva of subjects who are chewing the nut.

Arecoline can exist in both non-ionised and ionised forms in the mouth, with a pKa of 6.8, and therefore will become more ionised if the pH of the oral environment rises, which can occur if the subject uses slaked lime or smokes tobacco (Johnson and Bain, 2000). The study by Nair et al. (1985) indicated that higher levels of arecoline were found in subjects who used tobacco (Nair et al., 1985). This therefore presents an anomaly, for it seems that the higher the pH the more ionised it is and hence harder to cross the mucosal barrier. This may indicate that higher levels of arecoline were found in the mouths of those who had a more alkaline pH, due to the tobacco, and hence less of the arecoline remained in the non-ionised form available for absorption across the mucosal barrier, thus recording a higher level of arecoline in the mouth.

It would be interesting to investigate this further by examining arecoline levels in the plasma of subjects who use and who do not use tobacco and or slaked lime with the areca nut.
Arecoline Levels in Saliva

Arecoline was identified in the saliva from the parotid duct in subjects who had been instructed not to swallow any saliva during the period of the study. The resultant chromatograph demonstrated the presence of arecoline in the saliva from the parotid duct within the first minute, and concentration levels of the arecoline frequently 'shadowed' the levels found in the mouth but less, by a factor of approximately ten. These levels also appear with a time delay. However, there were some subjects who presented with arecoline in their saliva at time zero, indicating they had low levels of arecoline in their mixed saliva even before the study commenced. There are at least three possible explanations for this. Firstly, that the residual arecoline is possibly not secreted completely by the kidneys. The reason could possibly be due to the chemical structure of arecoline preventing movement of the drug into a high salt fluid such as urine, but instead establishing a 'steady state' equilibrium and allowing arecoline to move from systemic blood into saliva in the salivary glands, and from a tissue bank back into saliva. Secondly, it is possible that the non-ionized form of arecoline becomes sequestered in fat, and is slowly released into the circulation between chewing episodes. Finally, it is possible that arecoline is absorbed into dental plaque and slowly released, hence the level of arecoline in saliva might reflect the oral hygiene state of the patient.

This research demonstrates that arecoline has been detected in the saliva from the duct of the parotid gland. The concentration levels of the arecoline in the saliva is delayed compared to that found in the mouth, and the subsequent rise and fall in whole saliva is shadowed by the concentrations of arecoline observed in the duct saliva. In view of the fact that all participants in the study were advised to expectorate all saliva into the collection vials and not to swallow any saliva, then a reasonable explanation for the
Arecoline Levels in Saliva

presence of arecoline in the saliva in the duct of the parotid gland is that arecoline has been absorbed across the mucosal membrane of the mouth. Absorption of substances across the oral mucosal membrane has been described previously for other substances (section 3.1.2) and thus the absorption of arecoline across the oral mucosal barrier is probable.

This appears to be the first time that a substance absorbed across the oral mucosa has been documented to be present in the saliva. Previously entero-salivary recirculation has been observed with a number of substances including heavy metals and alkaloids (Lord et al., 2002; Richelmi et al., 1980; Wilhelm et al., 2002).

These results demonstrate that the concentration level of arecoline in the mouth varies significantly over time, if the subject spits out the juice of the areca nut. These levels are generally well above those required for stimulation of collagen synthesis, (0.1 µg/ml) as well as being cytotoxic for the cell (above 10.0 µg/ml). While the time period in the study was over a forty-five minute period it is accepted that the possibility exists for very high concentrations to be present in the mouth if the nut is used over extended periods of time, especially if the subject were to sleep with the areca in the mouth. Cytotoxicity would apply to fibroblasts as well as keratinocytes and endothelial cells.

The monitoring of the arecoline concentration levels, presented in this document, provides a dynamic picture of the fluctuation of these levels, with levels in the parotid duct saliva shadowing the levels found in the mouth, though greatly reduced. For the first time there is documentation supporting the idea that some individuals are more
Arecoline Levels in Saliva

'at risk' of developing an unusual response to the chewing of the areca nut, and that a small portion of the population that chew the areca nut can achieve a very high concentration level of arecoline. This could reflect a number of variables including pH of the saliva, rate of chewing of the areca nut, which in turn may reflect different personalities, stress levels. However, it was notable that there seemed to be a small sub-group of females who tended to have higher levels.

Unstimulated salivary flow rate is approximately 0.48 ml/min (SD 0.1-2.0 ml/min) (Fenoll-Palomares et al., 2004). This research demonstrates that the areca nut functions as a sialogogue. The mean value of the salivary flow rate for the 6 subjects in this study was 1.54 ml/min (SD 0.73) over three consecutive days. Once the nut was removed from the mouth the flow rate dropped to 1.05 ml/min (SD 0.41), but remained above the unstimulated salivary for the duration of the study.

Further research should explore these unknowns. Saliva samples should be taken from individuals who routinely use the areca quid at times when they are about to expectorate, to explore the levels of concentration normally reached by regular chewers. This research has identified individuals who appear to have a particular propensity to develop very high levels of arecoline on a regular and repeatable basis. The pH and buffering capacity of the saliva should be analysed in these individuals and to determine whether chewing patterns are a significant contributing factor. The mean concentration of arecoline in saliva is lower than expected. The highest concentration of arecoline in the mixed saliva of subjects in this study was 97.4 (SD 25.3) µg/ml, however this level was not sustained over any significant period of time.
Arecoline Levels in Saliva

The average of the highest concentrations during the active period (20 min of active chewing of the areca nut) in this study was 37.2 μg/ml of arecoline.

This may have implications for the laboratory studies that have been documented, where tissue is placed in high concentrations of arecoline for a long period of time. This situation does not reflect the general nature of the mouth, although it may reflect local areas immediately adjacent to the nut or betel quid.

In addition, this study demonstrates the reliability of the data, and for the first time demonstrates that individuals are unique in the manner in which they achieve a concentration of arecoline in the saliva. While Table 3.4 provides results that are similar to those of Nair et al (1985), Table 3.5 demonstrates that each individual achieves a level of arecoline concentration that is similar to their own previous studies but different from others (Nair et al., 1985).

Some factors that may contribute to the variation in arecoline concentration include salivary flow rate, the pH of the saliva, the rate at which the nut is masticated, the rate at which the arecoline is absorbed across the mucosal membrane. The results presented in Table 2 demonstrate the variability of the salivary flow rate amongst individuals as well as the significant variation in arecoline concentration, despite all individuals being given the standardized 0.5 gm sample of areca nut.

These results demonstrate degree of consistency within the one subject over the three day period. While the peaks in arecoline concentration may occur at slightly different times, the maximum concentration of arecoline obtained in the mouth, by any one
individual was similar for the three days. This was particularly if the concentration of arecoline was less than 20 μg/ml, with variation being less than 5 μg/ml. However, if the concentration was greater than 20 μg/ml then the variation was significant, and the period of time that the concentration was above 10 μg/ml was extensive.

One practical aspect of this study is that it provides a means of biochemically validating the data from the questionnaire and any intervention strategies undertaken in the future.
Chapter 4

A Clinical and Histopathological Investigation of Oral Submucous Fibrosis in Nepal
4.1. Signs and symptoms of oral submucous fibrosis

OSF is a chronic disease of insidious onset affecting the oral mucosa, with associated involvement of the pharynx and oesophagus. Both sexes are affected by OSF, with many studies showing a predominance of females (Gupta et al., 1980; Pindborg et al., 1968; Rao, 1962). The initial presentation of most patients is between 20 and 40 years of age; however, the age-range of people affected with OSF varies according to region (Gupta et al., 1980; Pindborg et al., 1980).

All clinical studies of OSF report the presence of constrictive fibrous bands, either in the buccal mucosa, the posterior part of the palate, or the labial mucosa (Ahuja and Agrawal, 1971; Akbar, 1976; Barnes and Duke, 1975; Chiu et al., 2002b; Cox, 1991; Dockrat and Shear, 1969; Dudani and Kher, 1971; Gupta et al., 1998; Hamner et al., 1974; Harvey et al., 1986; Jeng et al., 2001; Joffe, 1971; Lal, 1953; Mukherjee and Biswas, 1972; Pindborg, 1989; Seedat and van Wyk, 1988b). One of the main criteria for the clinical diagnosis of OSF is the presence of fibrous bands either in the buccal mucosa, the posterior part of the palate or the labial mucosa (Pindborg, 1989).

Reduced oral opening appears to be associated with stiffening of the oral mucosa and formation of fibrous bands (Haider et al., 2000). These fibrous bands run between the maxilla and mandible in the buccal mucosa, and are initially discrete, but gradually broaden and coalesce such that the whole of the involved mucosa becomes firm, with tissues assuming a leathery consistency. Involvement of the lips is characterized by the presence of fibrous bands running parallel but seemingly superficial to the orbicularis oris muscle. In the palate, the bands often radiate from the median raphe.
to the anterior faucial pillars. The faucial pillars usually become short and thick, and frequently the tonsils appear atrophic. When the soft palate is affected, its mobility is reduced, the voice becomes nasalized, and the uvula is often shrunken and bud-like. (Figure 1.2.D). Mucosal petechiae are described in about one fifth of OSF patients (Bhonsle et al., 1981).

There are regional differences in the presentation of OSF, and it has been postulated that these reflected the individual chewing habits of those who use the areca nut, including where the quid is held or the side where the nut is chewed, and whether the juice is spat out or swallowed (Bhonsle et al., 1987). In regions where the juice and quid are generally spat out, there is a higher involvement of the anterior sites of the mouth, compared with regions where the juices and quid are swallowed, in which case the posterior sites are primarily affected (Bhonsle et al., 1987). Dysaesthesia or a 'burning' sensation involving the oral mucosa is another common symptom associated with OSF, seemingly across all the regions where OSF is prevalent. This is in addition to reduced oral opening, associated with slowness and difficulty in eating, increased salivation, changed gustatory sensation, dryness of the mouth, and a nasalized voice (Chiu et al., 2002b; Chang et al., 2001; Huang et al., 1993).

4.1.1. The mechanism for induction of oral submucous fibrosis by areca nut use is not known

4.1.1.i. OSF is occasionally observed in non-chewers

The areca nut, is the fourth most common drug used worldwide, yet the incidence of OSF, allowing for geographic variations, remains relatively low at approximately
0.5% of the population using the nut (Canniff and Harvey, 1981; Winstock, 2002). Significant geographic variations are observed between countries as well as within national boundaries, with the incidence in India and China ranging from 0.05% to 1.4% and 0.9% to 4.7% respectively (Mehta et al., 1971; Zhang and Reichart, 2007).

OSF has traditionally been associated with chewing of the areca nut, however, some large population-based studies report individuals with OSF who have no apparent areca nut habit (Mehta et al., 1971; Seedat and van Wyk, 1988a). One study found for example, that only 77% of OSF patients used the areca nut in some form (Daftary, 1992). A further study involving 100,000 subjects reported that only 52% of OSF patients chewed areca nut. The same study demonstrated an incidence of OSF of 2.1% (Mehta et al., 1972). While areca nut use appears to have a strong association with the occurrence of OSF, the mechanism by which this develops is poorly understood.

A possible immune basis to the disease is suggested by a number of observations including altered lymphocyte subsets, a decrease in the total number of lymphocytes, and a reduction in cell mediated immune activity of both isolated peripheral blood mononuclear cells and lymphocytes infiltrating tumours in OSF patients (Chang et al., 2005a). It is possible that the areca nut exacerbates the immune responsiveness to some other, as yet unidentified, environmental agent responsible for OSF.

As noted in the earlier literature review (Section 3.1.1-2), although arecoline is toxic at high concentrations, it is also able to modify cell behavior at lower concentrations, and this is consistent with an indirect rather than direct role for the areca nut in OSF.
4.1.1.ii. Progression of oral submucous fibrosis is not well documented

Any discussion that considers the aetiological mechanisms for OSF, would have to consider whether the disease is progressive. An early histopathological description of OSF implied a progression through four stages, defined as 'very early', 'early', 'moderately advanced' and 'advanced' (Pindborg et al., 1964; Pindborg et al., 1965). Although this histopathological classification implies a predictable progression of OSF, there has been little documentation of OSF progression in individual patients. This may have clinical consequences, for although intervention therapies vary, the literature lacks any clear idea that different forms of therapy may be appropriate for different stages of OSF. In addition, the period of observation in most studies is very short and many studies appear to lack a control group (Maher et al., 1997; Pindborg, 1989). One objective of the work described in this chapter, was to determine if OSF affects oral tissue in a predictable order using a cross-sectional population approach, with a view to later longitudinal studies if appropriate.

4.1.1.iii. Possible aetiological mechanisms for oral submucous fibrosis

Chillies have been suggested as a possible aetiological agent in the development of OSF. A connective tissue response to capsaicin, the active ingredient of chillies, has been observed in Wistar rats (Sirsat and Khanolkar, 1960). Unfortunately, these observations could not be replicated by latter workers (Hamner et al., 1974) and no aetiological role for capsaicin was found in separate work comparing people with and
A Clinical and Histopathological Investigation of OSF in Nepal

without OSF with regard to the amount and the duration of consumption of chilies
(Wahi et al., 1966b).

Transforming growth factors – alpha and beta (TGF-alpha, -beta), known to stimulate
proliferation of fibroblasts in vitro, have been linked to OSF (Chiu et al., 2002a;
Haque et al., 1998; Hsu et al., 2001; Ma et al., 1995; Rajalalitha and Vali, 2005;
Tilakaratne et al., 2005; Trivedy et al., 1999; Vilcek et al., 1986). Enzymes associated
with the production and modification of collagen such as collagenases and lysyl
oxidase have also been suggested as playing a possible role in the development of
OSF (Chiu et al., 2002a; Ma et al., 1995; Tilakaratne et al., 2005; Trivedy et al.,
1999). Collagen-related genes are potentially modified by some of the components of
betel quid, while TGF-beta stimulates the transcriptional activation of pro-collagen
genesis in OSF (Rajalalitha and Vali, 2005). There is also correlation between
susceptibility for OSF and specific genotypes for collagen genes 1A1 and 1A2,
collagenase-1, TGF-beta, and lysyl oxidase (Chiu et al., 2002a). Over-deposition of
ECM (extracellular matrix molecules) is suggested as the basis for tissue fibrosis in
OSF (Chang et al., 2002). Complex interactions between cytokines have also been
suggested as possibly contributing to OSF, including activation of a TH2 type
immune response dominated by B cells, with exacerbated TGF-beta 1 release (Feng
and Ling, 2000; Snapper et al., 1993).

Malnutrition has been suggested as a contributing factor to OSF, as the disease
presents in many countries where malnutrition is rise. A greater incidence of poor
nutritional status is reported amongst people with OSF compared with controls (Wahi
et al., 1966a). Vitamin and iron deficiency states have also been implicated in the
A Clinical and Histopathological Investigation of OSF in Nepal

development of OSF. Marked iron and vitamin B complex deficiency has been observed in 77% of patients with OSF in Malaysia (Ramanathan, 1981). Nonetheless, the precise role played by these factors is unclear, while the possibility remains that an inability to eat because of OSF is the basis for the relative malnutrition, rather than malnutrition being causal of the disease.

Eosinophils, observed in connective tissues in OSF, have been suggested as indicating an allergic reaction (Pindborg and Singh, 1964), while an autoimmune basis for the disease has been further suggested by a number of workers (Canniff et al., 1985; Gupta et al., 1985; Haque et al., 1997; Pillai et al., 1992). However, some reject this concept as no specific allergen has been identified (Shah et al., 1994).

Nonetheless, increased levels of both local and circulating immune complexes have been observed in OSF subjects, and these have included complexes with IgA, IgG, and IgM (Balaram et al., 1987; Remani et al., 1988; Shah et al., 1994). Also, elevated serum IgG levels were observed among 30 OSF patients in England, relative to normal individuals (Canniff et al., 1986), while others have found higher levels of circulating immunoglobulin G, IgM and IgA in OSF patients (Gupta et al., 1985; Shah et al., 1994). Further support for a possible role for autoimmunity, comes from the observation that HLA A10, DR3, and DR7 are MHC phenotypes more often present in OSF subjects (Canniff et al., 1985). Nonetheless, while haplotypic pairs have been identified, extended studies have failed to demonstrate statistical significance for such associations (Chen et al., 2004; Chiang et al., 2002a; Chiu et al., 2001). Nonetheless, a possible relationship between OSF and the MHC class related
gene A (MICA) is reported, with a significantly higher frequency of the allele A6 of MICA in OSF patients compared to controls (Liu et al., 2004).

Other features of OSF that are consistent with a possible auto-immune aetiologic are the comparatively early age of onset, reported changes in circulating levels of serum immunoglobulins, the presence of autoantibodies, involvement of the DR locus in the genetic predisposition, and the female sex bias in many studies although this was not observed in Nepal (Adhvaryu et al., 1986; Borle and Jagtap, 1987; Canniff and Harvey, 1981; Canniff et al., 1985; Phatak, 1984; Rajendran et al., 1986).

Similar observations have been made in animals, with a possible immunological mechanism suggested by the observation that arecoline stimulates a delayed hypersensitivity response in mice (Selvan et al., 1991). Separately, a modulatory effect of arecoline on the B-cell mediated immune response is reported, with a reversible dose-dependent suppression of the immune response in rats (Selvan and Rao, 1993).

However, difficulties occur in interpreting some of these studies, in that controls were lacking in a study of serum auto-antibodies (Canniff et al., 1986), while circulating immune complexes noted as significantly increased in patients with OSF and oral cancer are also raised in healthy betel quid chewers compared with controls (Balaram et al., 1987). Similarly, elevated levels of circulating immune complexes, as well as of IgG and IgM, are observed in both oral cancer and OSF subjects (Remani et al., 1988).
4.1.2. **Histological features of oral submucous fibrosis**

4.1.2.i. **Leukocyte infiltrate associated with oral submucous fibrosis**

As mentioned above (4.1.1.ii.), four histopathological stages were originally described for OSF, and each of these has been characterized by a different profile of inflammatory cells (Sirsat and Pindborg, 1967). In this paradigm, the earliest presentation of the disease is accompanied by infiltrates of polymorphonuclear leukocytes, while lymphocytes and plasma cells become increasing prevalent as the disease progresses. The cellular infiltrate in OSF is composed primarily of T lymphocytes, and especially activated CD4+ helper/inducer T lymphocytes, while CD20 + B lymphocytes and CD68+ macrophages and Langerhans’ cells are only occasionally seen (Haque et al., 1997).

Of importance for the current study is that OSF is associated with the increased presence of antigen-presenting HLA-DR positive cells, as well as an increase in the ratio of helper to suppressor-cytotoxic T cells in both the epithelium and lamina propria (Haque et al., 1997). Similar observations are reported by others who note an increase in the numbers of T cells, B cells and CD68 positive macrophages with a predominance of CD4 lymphocytes over CD8 positive lymphocytes in the subepithelial connective tissue of OSF patients compared with controls (Chiang et al., 2002b). Interestingly, the infiltrate of T lymphocytes is reported to become less dense with progression of the disease from moderately advanced to advanced forms. However, when biopsies of moderately advanced and advanced OSF are compared,
there is no significant difference with regard to the infiltration of tissues by B-lymphocytes and macrophages (Chiang et al., 2002b).

4.1.2.ii. Epithelial changes associated with oral submucous fibrosis

Atrophy of the epithelium together with juxta-epithelial hyalinization is reported in OSF (Daftry, 1992), and these features are important when considering data shown in this chapter. An early report demonstrates that 70% of OSF biopsies show atrophic epithelium relative to controls, while about 2% have hyperplastic epithelium and the remainder have epithelium of normal thickness (Pindborg et al., 1970). There is loss of rete ridges in the majority of cases with atrophic epithelium, while rete ridges appear normal in the remaining specimens. In addition, the buccal mucosa, which is not normally keratinized, is ortho-keratinized in 26% of OSF biopsies, while 22% are para-keratinized (Pindborg et al., 1970).

'Signet-ring' cells, primarily in the basal epithelial cell layer, are reported in approximately 13% of OSF biopsies (Pindborg et al., 1970). However, interpretation of this data is difficult due to the absence of a control group in the relevant study (Pindborg et al., 1970), while similar changes can result from nuclear shrinkage as a fixation artifact in normal mucosa (Pindborg and Sirsat, 1966).

Pindborg (1970) likened the appearance of a number of biopsies to lichen planus, referring to the presence of 'colloid bodies' in the epithelium, a change that is now thought to be due to apoptotic keratinocytes. Other lichen planus-like features, noted by Pindborg (1970), were a tendency to formation of pointed rete ridges surrounded
by lymphocytes and a marked lymphocyte infiltration with a band-like distribution in the lamina propria. Nonetheless, on re-assessment, Pindborg et al. (1980) concluded that the OSF cases involved had co-existent lichen planus superimposed on separate and pre-existing OSF (Pindborg et al., 1980).

Signs of dysplasia in 7% to 25% of OSF biopsy specimens are important to note from the perspective of the malignant potential of the disease (Pindborg et al., 1966; Pindborg et al., 1970). It is interesting to note differences in the features of dysplasia associated with OSF compared with those found in leukoplakia (Mehta et al., 1969). In leukoplakia, the main dysplastic characteristics are increased mitotic activity, hyperchromatism and basal cell hyperplasia, whereas in OSF, basal cell hyperplasia is rare and irregular epithelial stratification, nuclear pleomorphism and a pronounced intercellular oedema are the predominant dysplastic features (Mehta et al., 1969). This is consistent with earlier findings in OSF where an increased number of mitotic figures, nuclear hyperchromatism and loss of cellular polarity were noted as prominent dysplastic features (Pindborg et al., 1970). In particular, emphasis has been placed on the presence of prominent intercellular oedema, especially in the basal cell layers, which is reported as occurring in 75% of epithelial dysplasias (Pindborg et al., 1970), while it is unfortunate that there was no control group in this particular study.

4.1.2.iii. Change in the lamina propria in oral submucous fibrosis

The first description of the changes in the lamina propria in OSF was published by Sirsat and Pindborg (1967), who also proposed the four different histological stages mentioned above. The first or 'very early' stage is characterized by finely fibrillar
collagen, marked oedema, large fibroblasts and blood vessels that are often dilated and congested. The inflammatory exudate consists mainly of polymorphs and occasional eosinophils. The 'early' stage follows and is associated with early signs of hyalinization in the juxta-epithelial region, in which collagen bundles are thickened and there are moderate numbers of fibroblasts. At this stage the inflammatory cells are mainly lymphocytes, eosinophils and plasma cells. In 'moderately advanced' and 'advanced' stages, there is a variable extent of hyalinization of the connective tissue. No oedema is reported for the 'advanced' stages, while the predominant inflammatory cells are lymphocytes and plasma cells and the state of the blood vessels in the 'moderately advanced' stage is reported as ranging from normal to being constricted, while there is obliteration of vessels in the 'advanced' stage (Sirsat and Pindborg, 1967).

4.2. Materials and Methods

This study was part of a prospective study developed to examine the success of various treatment regimes in the management of the loss of oral opening in patients with OSF.

4.2.1. Collection of clinical and demographic data

4.2.1.i. Development of OSF clinical questionnaire

A questionnaire was developed for the purposes of documenting the demographic data of patients with OSF, as well as the characteristic features for all patients presenting with OSF. Information on the maximal oral opening and clinical signs and symptoms was collected for all patients, although in some cases not all clinical
features of interest were recorded. A questionnaire (Figure 4.1) was designed to collect data in relation to four main areas relating to demographics, oral habits, the site of presentation of the disease and the clinical signs and symptoms associated with the disease.

Using a similar approach to that described in Chapter 2 (2.2.1.ii.), an earlier draft of the questionnaire was first tested with a number of clinicians and patients. This resulted in slight modifications, including provision for recording the presence of ulceration and ‘blistering’.

4.2.1.ii. Mucosal biopsies and histopathological confirmation of the disease

Permission for an incisional biopsy of the mucosa affected by OSF was sought from all patients who presented with the clinical signs of the disease. Informed consent was obtained only after appropriate information regarding the technique and negative and positive sequelae of the procedure were communicated to the patient. This included the information that the clinical symptoms would worsen for a limited period of from 4 to 12 weeks due to the surgical stimulation of fibrosis. Thirty-seven (61%) of the patients presenting consented to an incisional biopsy under local anaesthetic. All biopsies were taken at Patan Hospital, Kathmandu, from patients who were clinically diagnosed as having oral submucous fibrosis.

Blood was also collected from 34 patients (55.7%) consenting to this additional procedure, for determination of full blood count, haemoglobin and haematocrit values.
**Figure 4.1**

Form used to gather demographic and clinical data on subjects in Nepal who presented with signs and symptoms of OSF. The form allowed for the appropriate documentation of treatment as described in Chapter 5, and the progression of clinical signs and symptoms in a visual and numerical form. Symptoms as well as clinical signs were registered initially and on subsequent visits. Oral opening as measured in mm between two opposing central incisors using a vernier caliper was recorded. ‘Affected Areas’ related to the mucosal areas with fibrosis, while ‘pain’ was recorded as either being present all the time, or only while eating. ‘Blistering’ and ulceration related to the clinical presence of vesicles at the time of examination. ‘Active opening’ and ‘injections’ related to the treatment trialed as detailed in Chapter 5.
## Oral Submucous Fibrosis

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4.2.1.iii. Recording of clinical signs and symptoms

Patients presented to Patan Hospital (Kathmandu, Nepal) complaining of limited oral opening. The study extended over a period of 5 years and involved 61 patients. Ethical approval was from the Ethical Committee of Patan Hospital. Trained investigators interviewed patients and recorded: gender, age, areca nut habit, and occurrence of pain; and also examined patients for inter-incisal distance, identification of sites with OSF confirmed by biopsy, and oral vesicles and or ulcers. Blood tests for anaemia were offered, but accepted by only 34 participants. Unfortunately, in consequence of the difficulties associated with engagement of multiple examining clinicians, of the 61 participants, a pain history was only recorded for 40 subjects, while only 31 had further records made of the presence or absence of oral ulceration or vesicles. Nonetheless, sufficient data was collected to permit meaningful statistical evaluation by Wilcoxon’s Ranked sign, Mann-Whitney U, and Chi-square tests.

Inter-incisal distance was determined in two recordings per visit between left maxillary and mandibular central incisors, or the nearest appropriate teeth if the incisors were absent. Measurements were always passive without force applied. 32 mm was recognized as the lower limit of normal inter-incisal distance in the Nepali population (Cox and Walker, 1997).

Patients were encouraged to attend for review every month, and support of the patients. This regime was only occasionally adhered to, and then by those who lived within easy traveling distance of the hospital.
4.2.2. **Biopsy materials and histological assessment**

Biopsy specimens were placed in a 10% neutral buffered formalin solution (Shirdi Industries Ltd, Mumbai, Maharashtra, India) for 12 hours prior to dehydration with graded alcohols and histoclear (Apratin International, New Delhi, India), followed by infiltration with paraffin (Unique India, New Delhi, 110026 India) for section microscopy. 5μm sections were prepared with an ‘Advanced rotary’ microtome (Rattan Sales Corp., Model Basti, New Delhi 5, India), and collected onto glass slides (Proteq Systems, Shahdara, Delhi, 110032 India) before rehydration with histoclear and graded alcohols, and subsequent staining with haematoxylin and eosin (Belami Fine Chemicals Pvt. Ltd. Mumbai, India). Sections were examined using a Coslab Binocular microscope (Coslab, Ambala Cantt, Haryana India), and histopathological features recorded. To help control for possible observer bias, sections were examined by a separate pathologist on two different occasions.

The histological reports were undertaken by Assoc Prof Hedley Coleman and Assoc. Prof Hans Zoellner.

4.2.3. **Statistical analysis**

The statistical software package SPSS Graduate Pack Version 13.0 for Mac OS X was used to analyze the data. The Chi-square test was used to evaluate the statistical significance of differences in proportion, while the Mann-Whitney U Test was also used where appropriate. Two-tailed tests were used throughout with statistical significance being accepted in instances where p < 0.05.
4.3. Results

4.3.1. Oral Submucous Fibrosis achieved Peak

Prevalence by 20 to 40 Years of Age

Sixty-one patients were included in the study, being 19 females and 42 males. The participants in this study were not included in the epidemiological study described in Chapter 2. The age of participants ranged from 6 to 77 years, with an average of $35.5 \pm 14.9$ years, females and males being $33.4 \pm 14.1$ years and $36.7 \pm 15.2$ years respectively, while the slight difference between sexes was not statistically significant. Figure 4.2 demonstrates the sex distribution according to age group of the participating OSF patients. Fifty-one percent (31/61) of the OSF cases in this study fell in the 20-40 year age group.
Figure 4.2

Histogram demonstrating the proportion of males (black bars) and females (white bars), according to decade, presenting with OSF in the current study. While the male to female ratio for OSF was 2.2:1 the relative incidence of the disease across age groups was similar. The age-group of 20-29 years had the highest proportion of OSF cases amongst both males and females.
4.3.2. Regular areca nut use was prevalent but not universal amongst people with oral submucous fibrosis

The majority of the patients with oral submucous fibrosis, 91.8% (56/61), indicated they used areca nut with or without tobacco, with 78.9% of females and 82.1% of males reporting an areca nut habit. Importantly, this left 8% (5/61) of patients, comprising 4 females (mean age of 42.8 ranging from 32 to 52 years) and 1 male aged 40 years, indicating they did not use the areca nut and had not done so in the past. Although there were only five people in this group, the average oral opening for this group was 32.7 mm (SD 13.8 mm), so that oral opening was less restricted than amongst those patients reporting areca nut use, and this difference was significant (p<0.04).

The majority of the 61 OSF patients (63%) indicated they had commenced use of the areca nut during childhood, with only 3 subjects (5%) indicating they commenced the habit in their teens or later. Separately, there were a large number of individuals (32%) who were unable to recall when they commenced using the areca nut, consistent with likely use during earlier childhood.
4.3.3. **Presentation of clinical signs associated with OSF**

4.3.3.i. **Oral submucous fibrosis affected different mucosal surfaces in a predictable order and this correlated with reduced oral opening**

OSF presentation in Nepal appeared to affect discrete mucosal surfaces in a predictable way, such that the soft palate was first affected, with progressive disease involving the fauces, the buccal mucosa unilaterally and then bilaterally and then eventually the floor of mouth. In extremely advanced disease with severely restricted oral opening, the labial mucosa was affected as well. Figure 4.3 shows the way that increased oral mucosal involvement correlated with reduced oral opening. When the oral opening of patients with relatively early disease affecting the soft palate, fauces or unilateral buccal mucosa, was compared with that in patients with more advanced disease, a strong relationship between advanced disease and reduced oral opening was seen ($p < 0.006$). The disease always appeared to progress in this order in the Nepali population studied. However, in no situation was the disease actually observed to progress in any of the patients during the period of the study.

The age and maximal inter-incisal distance of individual patients with OSF is plotted and marked according to sex in Figure 4.4. The mean value for the inter-incisal distance for these 61 individuals was $26.9 \pm 10.2$ mm. Females presented with an oral opening of $23.4 \pm 10.8$mm, and males $28.4 \pm 9.8$ mm, while the difference between means for both sexes was not statistically significant. No clear relationship between
age and reduced oral opening was found.

4.3.3.ii. The major clinical symptoms of oral submucous fibrosis were restricted oral opening and pain

While all 61 OSF patients reported a subjective history of reducing oral opening, 51 (82%) presented with an inter-incisal distance of less than 32 mm considered the lower limit of normal opening in the Nepali population. Twenty-one out of the 40 patients for whom a pain history was available reported that they had oral mucosal pain, comprising a pain incidence of 53% of patients with OSF. Although it might be expected that there would be a correlation between the severity of pain and degree of restriction in oral opening, no clear relationship between these two clinical features of OSF was seen (Figure 4.5). Similarly, there was no statistically significant difference between the sexes with regard to the incidence of pain or opening less than 32mm. Nonetheless, 15 out of these 40 patients (37%) reported they had pain during eating, while 6 out of 40 patients recorded indicated almost constant pain (15%). It was not possible to clinically distinguish between the painful symptoms described by the OSF patients and those symptoms associated with ‘burning mouth’ syndrome.

Interestingly, not all patients with vesicular or ulcerative lesions reported a history of pain, such that 6 out of 10 people with vesicular lesions, comprising 2 females and 4 males, did not volunteer a history of oral pain. There was no statistically significant difference between the sexes with regard to the incidence of pain or distribution of vesicular or ulcerative lesions.
The presence of vesicular or ulcerative oral lesions was recorded for only 31 of the OSF patients studied, but of these 11 (35%) presented with one or other of these lesions. Vesicles were seen in 3 of 7 females (42%), and 7 of 24 males (29%), so that considering both genders together 10 of the 31 OSF patients for whom the presence or absence of ulcerative or vesicular lesions was recorded (32.3%), had vesicular lesions on examination. Ulceration was seen in 1 of 7 females (14%) and 6 of the 24 males (25%) recorded, so that between the two sexes 7 out of 31 OSF patients had ulcerative lesions (22.6%). While vesicular lesions were more frequent compared with ulcers, the two types of lesions usually occurred together, such that only one of the patients with oral ulceration did not also have vesicular lesions.
Figure 4.3

*Scattergram showing oral opening according to the progressively increasing oral mucosal surfaces affected with OSF. OSF appeared to affect the oral mucosa in a highly predictable way, such that fibrosis affected first the soft palate, and then the fauces, unilateral buccal mucosa, bilateral buccal mucosa, floor of mouth and lips in that order. This was accompanied by progressive reduction in the inter-incisal distance (p < 0.006), with most patients presenting with an oral opening less than 32 mm (dashed line) being the lower end of the normal range of opening in Nepal.*
Figure 4.4

Scatter diagram indicating the age, sex and oral opening (mm) in individuals at the time of presentation relative to the lower limit of normal opening in Nepal (dotted line). Eighty four percent of patients presented with an inter-incisal distance below the lower limit of normal opening in Nepal. There was no clear relationship between the age of presentation or oral opening, while there was similarly no clear difference between the sexes.
4.3.3.iii. An absence of correlation between haematocrit and oral opening in oral submucous fibrosis

Of the 61 participants, 34 agreed to have blood tests being 10 females and 24 males, of whom 6 females (60% of those with blood tests) and 7 males (29% of those with blood tests) were found to have anaemia. Females had mean values for packed cell volume of 33.5%, with a range range of 17% to 43% (normal 35% to 45%), and males had a mean packed cell volume of 40%, ranging from 13% to 50% (normal 40% to 50%). Figure 4.6. illustrates the haematocrit values obtained for individual males and females, relative to oral opening. No clear association between haematocrit and oral opening or gender was seen.
Figure 4.5

Scattergram showing the inter-incisal distance of individual OSF patients grouped according to whether they had no pain, pain on eating or constant pain. Although there was substantial variation in oral opening, with many patients having an inter-incisal distance of less than 32 mm considered the lowest opening in the normal range, there was no convincing correlation between limited oral opening and the severity of pain suffered by patients.
Figure 4.6

Scatter diagram plotting haematocrit for male and female participants with OSF relative to oral opening. The lower limits of the normal ranges of haematocrit for Nepali males (dotted line) and females (dashed line) are shown. The lower limit of the normal range of oral opening is also indicated (bold arrow). No clear relationship between haematocrit and oral opening was seen.
4.3.3.iv. Four OSF patients also presenting with oral squamous cell carcinoma (OSCC)

Of the 61 patients in the study, four patients (one female and three males) were identified with oral carcinomas, with the mean age being 46.3 ± 9.9 years. Three of these presented with OSCC at the time of first examination, while one patient developed OSCC fourteen months after initial presentation and clinical confirmation of OSF. This last patient was female, did not report any oral habit and was the youngest presentation of OSCC at 33 years, while the other three were males and reported regular use of the both areca nut and tobacco in the form of cigarettes or bidis. The four OSCC cases all had moderately to well-differentiated squamous cell carcinomas. Two of these presented on the buccal mucosa, another on the posterior maxillary gingiva, and the fourth occurred in the vestibule of the lower lip, where a preparation of areca nut, tobacco and spices was habitually held. Figure 4.7 shows the clinical appearance of one of these patients, as well as the attendant histopathology of the invasive tumour.
Figure 4.7

Clinical photograph and histopathological images of a male patient, 60 years of age, with OSF and OSCC, and who smoked tobacco and chewed areca nut. (A) The carcinoma occurred in the buccal mucosa but was largely obscured by a dense fibrous band (FB) stretching between the attached gingiva of the maxilla and mandible. (B) Histopathology of the malignancy revealed invasive islands of well differentiated keratinocytes (arrows) below the level of the epithelium (Ep), associated with a dense inflammatory infiltrate (In). (C) Mild dysplastic changes were seen in adjacent epithelium, with drop shaped rete ridges (arrow heads) and infiltrates of lymphocytes and plasma cells (In) together with early juxta-epithelial hyalinization characteristic of OSF. (Bars, B = 250 µm, C = 50 µm)
4.3.4. **Histopathological features of oral submucous fibrosis observed in the Nepali population**

4.3.4.i. **Confirmation of the clinical diagnosis of oral submucous fibrosis**

Thirty-seven formalin fixed, and paraffin embedded biopsy specimens were available for analysis, with 57% of patients in the 20 to 40 year age group. The characteristic features of OSF are sub-epithelial deposition of dense and hypovascular collagenous connective tissue, chronic inflammatory cells to a varying degree, epithelial atrophy or hyperkeratosis in early lesions, and epithelial dysplasia. These features were seen in all biopsies studied, although not all features were seen in all cases. Interestingly, there was little correlation between clinical symptoms and the histological stage of the disease as defined by Sirsat and Pindborg (1967). Further, in the five cases where multiple biopsies were obtained from the same patient, histopathological diagnosis was of different stages on the basis of the published staging method (Sirsat and Pindborg, 1967).

The appearance of collagen was variable, particularly in the sub-epithelial area. In 2 out of 37 specimens (5%), collagen had a finely fibrillar appearance and there was no hyalinization of collagen while in a further 12 cases (32%), thickened bands of collagen with early signs hyalinization in the juxta-epithelial area were seen. Five of the remaining 23 cases demonstrated a complete band of hyalinized collagen in the juxta-epithelial region where no clearly separate bundles of collagen could be discerned (Fig. 4.8). Six percent of the biopsies revealed all three collagen patterns in different parts of biopsies, often less than 8mm apart. Surprisingly, there was no clear
association between the histopathological presentation, and age, nor the length of time that subjects reported exposure to areca nut.

Inflammatory cells were present in all specimens studied, with 4 cases of 37 cases having at least some neutrophilic polymorphonuclear leukocyte infiltrates with occasional eosinophils, while the remaining 33 cases had infiltrates comprising primarily lymphocytes and some plasma cells. In 13 cases, infiltrates of lymphocytes were diffuse, while in 3 cases were patchy. The remaining 12 cases had a dense band-like infiltrate of lymphocytes, reminiscent of lichen planus (Figure 4.8).

There was some degree of correlation between leukocytic infiltrate and clinical symptoms, in that oral opening was in the normal range for those cases where primarily polymorphonuclear cells were present. Oral opening of less than 32mm was seen in all cases where collagen hyalinization occurred, and this was typically associated with lymphocytic infiltrates, though one case was markedly acellular with severe hyalinization. A further feature expected from the literature, was melanin incontinence in 13 out of 37 cases studied (Figure 4.8).
Figure 4.8

Paraffin sections of specimens from patients with OSF, displaying features characteristic of the disease. Stratified squamous epithelium (Ep) was present in all biopsies examined, while normal delicately reticular juxta-epithelial collagen was present in parts of individual biopsies from most patients (A). (B) Partial hyalinization (PH) was seen in many specimens with apparently advanced disease. Infiltrates (In) of primarily lymphocytes with plasma cells and occasional eosinophils (Eo) were seen in the lamina propria of all specimens studied, often beneath areas of juxta-epithelial hyalinization (JEH), while the extent of these inflammatory cell infiltrates varied from small clusters (Cln) to extensive broad sheets of cells (C,D,E,F): (G) Melanin incontinence was frequently seen (arrow heads). (H) In apparently advanced disease, partially hyalinized collagen extended beneath broad zones of juxta-epithelial hyalinization, often associated with a thin and atrophic epithelium. (H&E, Bars = 40 μm)
4.3.4.ii. Novel histopathological features observed in oral submucous fibrosis

In addition to the histopathological features expected for oral submucous fibrosis outlined above, a number of features were noted which had not apparently been described in the earlier literature.

In 4 out of 37 cases, inflammatory infiltrates had clearly perivascular and perineural distributions, while in 3 specimens a mixed inflammatory cell response containing neutrophilic polymorphonuclear leukocytes was seen in minor salivary glandular tissue (Figure 4.9).

An unusually close association between capillaries and the overlying epithelium was noted in 5 out of 37 cases, so that the endothelial lining of capillaries were apparently in direct contact with the epithelium, and in 2 further cases appeared to enter the epithelium from the underlying lamina propria (Figure 4.9). This last feature was apparent only in cases with features histologically defined as ‘early’ OSF, where there was increased vascularity and minimal reduction in oral opening. In addition, perivascular collagen appeared at least partially hyalinized in 33 cases (Figure 4.9). Infiltration of the epithelium by lymphocytes was sometimes very extensive, so that the epithelium became heavily vacuolated and indistinct (Figure 4.9). At times, this appeared very similar to the basal cell liquefaction, characteristic of lichen planus, and this was noted in 30 out of 37 cases studied, together with infiltration of lymphocytes into the basal cell area (Figure 4.10). Importantly, despite this appearance, as well as the frequent but usually not coincident presence of a lichenoid band-like infiltrate of lymphocytes, no patients presented with the clinical signs of lichen planus.
Intra-epithelial vesicles were noted in specimens of 3 of the 37 cases biopsied. These were often associated with intra-epithelial infiltrates of lymphocytes, while occasional rounded Tzank-like cells were seen (Figure 4.10). It was of note that nine out of 61 patients studied reported vesicles in the mouth, usually after eating spicy foods.
Figure 4.9

Paraffin sections of OSF specimens displaying apparently novel histopathological features. Vessels (V) were readily identified and often contained erythrocytes while these were occasionally surrounded by dense inflammatory infiltrates of lymphocytes (In) (A), as were some nerves (N) (B). (C) Minor salivary gland tissue with mucous acinar cells (Ac) occasionally had a mixed inflammatory infiltrate with numerous neutrophilic polymorphonuclear leukocytes (PMN). (D) An unusually close relationship between superficial vessels (V) and the stratified squamous epithelium (Ep) was seen in some specimens, such that there was intimate contact between endothelium and epithelium (D, G). (E) Collagen about some vessels (V) was hyalinized. (F) Heavy epithelial lymphocytic infiltrates were associated with vacuolation of the epithelium (VEp), and this may have been the origin of intraepithelial vesicles (Ve) (G,H), where an acantholytic process appeared to result in the appearance of occasional free-floating Tzank-like cells (arrows) (H). (H&E, Bars = 40 μm)
Figure 4.10

Paraffin sections of OSF biopsies demonstrating basal cell liquefaction and collagen hyalinization. (A) Heavy infiltrates (In) of lymphocytes with plasma cells and occasional eosinophils (Eo) were seen. (A,B) At times, the lymphocytic infiltrate extended into the adjacent epithelium (Ep), with the appearance of basal cell liquefaction (BCL) similar to that seen in lichen planus. The vacuolation and infiltration was not always confined to the basal layers, with spinous cells also sometimes involved (C). (D) Despite the unexpected epithelial degeneration seen, other areas of these biopsies had the partially hyalinized collagen (PH) expected of OSF. (H&E, Bars = 40 μm)
4.4. Discussion

The present study included histo-pathological confirmation of OSF, while the main clinical and histopathological features found were consistent with those reported by others (Pindborg and Sirsat, 1966; Pindborg et al., 1980; Seedat and van Wyk, 1988a; Sirsat and Pindborg, 1967). Nonetheless, several novel observations were made in the Nepali population studied. Of some interest, was that there was no clear relationship between the clinical signs noted and the histopathological presentation, with different biopsy sites in the same patients displaying features considered representative of different stages of the disease (Sirsat and Pindborg, 1967) (Section 4.3.4). This suggests that the ‘staging’ proposed by Sirsat and Pindborg (1966) may require revision, although a separate interpretation consistent with the literature is that the disease progresses at different rates to achieve different stages in different parts of the same mouth. This would be consistent with the apparent progressive involvement of consecutive mucosal tissues. However, this can only be confirmed with a longitudinal study and while notably the current work describes, in part, such a progression a study of a longer duration is necessary to confirm these initial observations.

The photomicrographs and descriptions of OSF published by various authors including Sirsat and Pindborg (1966), do not seem to completely encompass the histopathological presentation observed in Nepal and it is possible that this reflects geographic variations of the disease. Such geographic variations could be due to multiple factors including genetic variations, diet, other immunological challenges to the patient from parasitic infections, or malnutrition, as well as differences in customs for areca nut use. It would be interesting to establish a further multi-centre study to extend the current observations with larger numbers of patients from different areas,
including communities with known different patterns of areca nut use. The use of
standard protocols would facilitate identification of true regional variations in disease
presentation, and potentially lead to a greater insight into possible aetiological factors.

The current study was hampered by a suboptimal number of patients for study, and
this was particularly so with regard to patients who denied areca nut use. These
patients challenge the general assumption that areca nut is the primary cause of OSF,
and raise the possibility that areca nut may accelerate OSF initiated by some other as
yet unidentified cause, or alternatively that the disease may be caused by multiple
independent agents. This may be consistent with an immunological basis for OSF, as
areca nut may in some way promote an immune response to separate directly causal
agents, despite the nut being perhaps not directly causal of the disease.

Several workers have suggested an immune basis for OSF (Canniff et al., 1985;
Chang et al., 2005b; Liu et al., 2004). The novel observations made in this thesis, of
histopathological features similar to the well recognized immune disease of lichen
planus (Section 4.3.4.), support a role for the immune system in OSF. With regard to
this, it is important to note that despite some histological similarities with lichen
planus, none of the patients studied had the clinical features of lichen planus, while
none of the biopsies studied had the classical features of this immune disease but were
instead more consistent with the well accepted features of OSF (4.3.4.i.). In light of
this, it seems possible that Pindborg’s earlier report of patients suffering with both
OSF and lichen planus may not have represented two super-imposed pathologies in
identical sites (Pindborg and Sirsat, 1966), but instead simply reflected similar
underlying pathological processes in both diseases, as suggested in the current study.
Similarly, the perivascular and perineural lymphocytic infiltrates observed in this thesis, raise the possibility that vascular and or neural immune mediated damage may play a central role in the avascular nature of OSF, as well as the pain suffered by these patients. It is interesting that there was no clear correlation between pain and oral opening in the current study, suggestive that perhaps pain is elicited by mechanisms independent of the fibrotic response.

One benefit of the current study was that it provided the opportunity to examine the histopathological features of OSF in individuals from a relatively limited ethnic community. The geo-political features of Nepal, with its high mountain ridges running east-west across the country, sandwiched between the ice covered Tibetan plateau, and the Gangetic plains of India, has limited the free movement of the population.

It would be very interesting to develop a longitudinal study to examine whether OSF progresses in a predictable manner, as was suggested by the data in this cross-sectional study. An expanded multi-centre study would be able to provide adequate information.

It would also be interesting to characterize the specific sub-sets of lymphocytes in the biopsies studied. An attempt to do this was made in the current thesis, however, the cellular antigens were insufficiently preserved in biopsies for proper characterization of lymphocytic infiltrates. Nonetheless, it would be interesting to perform further work, using frozen section immuno-histochemistry to better define the cellular populations involved.
Finally, the data presented here for Nepal does not support the reported female predisposition to OSF (Gupta et al., 1980; Maher et al., 1994; Murti et al., 1990) reported in many centres, although there are studies which also go against this trend (Hazare et al., 1998). The male to female ratio of 2.2:1 amongst those seeking assistance and attending Patan Hospital is consistent with the epidemiological data presented in Chapter 2, where all the individuals with OSF were male.
Chapter 5

Trial of a Physiotherapeutic Strategy for the Management of Oral Submucous Fibrosis
5.1. Introduction - physiotherapeutic strategy for the management of OSF

The principal clinical problems for sufferers of oral submucous fibrosis relate to reduced oral opening with associated difficulties in eating, and severe mucosal discomfort. There are numerous anecdotal stories of patients spending hours trying to eat a meal, an outcaste in the family. Other signs include mucosal lesions, nasalised voice and sensitivity to spicy foods as well as hot substances. Malnutrition is common in many parts of the world, and is also commonly seen in OSF. The possibility remains that OSF contributes to malnutrition by limiting the opportunity to eat, while there may also be a role for malnutrition in development of OSF itself (Borle and Borle, 1991; Lai et al., 1995).

5.1.1 Philosophies behind different treatment regimens for oral submucous fibrosis

While it is clear that OSF is primarily a fibrotic condition, it seems reasonable to consider this as reflecting imbalance in the normal homeostatic synthesis and degradation of extracellular matrix. Chronic inflammation appears a dominant factor in creating this imbalance, implicating a role for cytokines and growth factors (Goldring and Goldring, 1991). Transforming growth factor-beta (TGFβ) is recognized as important in driving fibrotic responses (Duncan et al., 1999; Illsley et al., 2000; Rajalalitha and Vali, 2005), stimulating matrix synthesis (Frazier et al., 1996; Hong et al., 1999; Rajalalitha and Vali, 2005), and also in inhibiting matrix metalloproteinase synthesis (Rodland et al., 1990). Other growth factors implicated in fibro-proliferative disorders are platelet-derived growth factor (PDGF), basic
fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-1) (Bienkowski and Gotkin, 1995).

As detailed below, this has led to attempts to intervene with the disease process using cytokines, steroids and even hyaluronidase to modify the inflammatory and or fibrotic process (Borle and Borle, 1991). An alternative strategy has been more mechanical in its approach, using surgery to relieve constricted tissues and reconstruct the oral cavity. It is perhaps not surprising that this surgical strategy which ignores the underlying cause of disease, has had the least success, but none-the-less it is equally disappointing that seemingly more biologically based treatments have also had only limited efficacy in management of oral submucous fibrosis (Canniff et al., 1986; Khanna and Andrade, 1995; Paissat, 1981). Combinations of surgery with pharmacological and or physiotherapeutic approaches have also had a chequered success (Katharia, 1994; Kumar et al., 2007).

5.1.2. Treatment regimes used in the management of oral submucous fibrosis

5.1.2.i. Surgical approach to the management of oral submucous fibrosis

Surgical intervention is generally directed at the relief of acute symptoms and therefore the procedures range from the removal of front teeth to facilitate eating, to the surgical removal of fibrous bands, with or without surgical reconstruction of the tissues.
The surgical removal of fibrous bands from the buccal mucosa is ineffective and unfortunately detrimental. Clinical experience demonstrates that additional trauma, in the form of surgery, leads to even further contraction of the tissue, and an exacerbation of clinical symptoms (Le et al., 1996; Mokal et al., 2005).

Varying success has been had in the sectioning of fibrous bands combined with the interposition of tissue flaps and grafts from otherwise unaffected sites, including the tongue, naso-labial tissue, forearm flap, and even use of lingual pedical flaps (Hosein, 1994; Kavarana and Bhatnana, 1987). Unfortunately, short-term success has normally been followed by relapse, especially if the chewing habit has been maintained (Le et al., 1996; Mokal et al., 2005). A comparative study undertaken by Lai et al. (1995) suggested that surgery was successful in subjects whose inter-incisal distance was less than 20mm (Lai et al., 1995). Unfortunately there was no randomisation of treatment modalities and there were very few females in this study.

It is interesting to note that skin grafts become fibrosed in a high proportion of patients who continue the areca nut habit, suggesting that the disease may not be fundamentally an oral mucosa lesion, but rather is reactive to some component of the areca nut (Meghji and Warnakulasuriya, 1997).

Recently, Mokal et al. (2005) advocated extensive surgery releasing the masseter muscle from the zygoma, as well as the temporalis muscle from the coronoid process and mucosa, and the buccinator muscle with pterygomandibular raphe from their respective insertions. This was combined with the inter-placement of the temporalis muscle into the defect and covering the intra-oral area with a split skin graft from the thigh (Mokal et al., 2005). This is advocated as providing long-term relief from
trismus (Mokal et al., 2005), but detailed data on this are presently not available. In light of the earlier observations (Meghji and Warnakulasuriya, 1997), it seems likely that unless the causative oral habit ceases, even this highly intensive surgical intervention would be followed by recurrence of OSF. Of interest, are improved outcomes with post-surgical stents or physiotherapy (Lai et al., 1995; Le et al., 1996).

5.1.2.ii. Pharmacological approaches to the management of oral submucous fibrosis

5.1.2.ii.a. Steroids and hyaluronidase in the management of oral submucous fibrosis

The weekly use of topically applied or submucosal intralesional steroid injections has been advocated as a treatment regime in controlling the progress of the disease (Borle and Borle, 1991). However, this form of steroid treatment alone has had only limited success, with minimal improvement (Borle and Borle, 1991; Lai et al., 1995).

An alternative approach to management of OSF is based on the assumption that hyaluronic acid and matrix proteins play a central role in the organization of the extracellular matrix and involves local injection of hyaluronidase and or chymotrypsin (Gupta and Sharma, 1988; Kakar et al., 1985). Unfortunately, while rapid results have been obtained with this approach, the benefits have been limited and the combined therapy of hyaluronidase and steroids was associated with better, although minimal, long term results over a 2 year period (Borle and Borle, 1991; Chaturvedi, 1989; Gupta and Sharma, 1988; Kakar et al., 1985; Lai et al., 1995). Importantly, Borle and Borle (1991) indicated that although there was no improvement in oral opening following treatment with injections of steroids and
hyaluronidase compared with topical Vitamin A, steroid applications, or oral iron preparations, there was improvement in other symptoms including the burning sensation and stiffness of the oral mucosa, with disappearance of vesicles, although these returned within three to four months (Borle and Borle, 1991).

5.1.2.ii.b. Use of a placental extract to manage oral submucous fibrosis

Placental extract has been described as having anti-inflammatory effects (Anil and Beena, 1993; Kakar et al., 1985; Katharia, 1994; Sharma et al., 1987; Sur et al., 2003). Consistent with an inflammatory or immune basis to oral submucous fibrosis, cessation of the areca nut habit and submucosal delivery of an aqueous solution of placental extract is reported to produce a marked improvement in the disease (Anil and Beena, 1993; Kakar et al., 1985; Katharia, 1994; Sharma et al., 1987).

5.1.2.ii.c. Intra-lesional injection of gamma interferon in the management of oral submucous fibrosis

The anti-fibrotic properties of the cytokine Interferon-gamma (IFN-γ) have been utilized in the immuno-regulation of the symptoms of submucous fibrosis (Haque, Meghji, et al. 2001). Improvement was noted in subjects given 50μg of intralesional INF-γ; the subjects were asked to undertake stretching exercises as well as to cease the chewing habit (Meghji and Warnakulasuriya, 1997). This treatment resulted in significant improvement (Haque et al., 1998) with an overall mean net gain in inter-incisal distance of 8.4 mm ± 3.4 mm, an improvement of 42% ± 18%. However, the financial cost of this treatment is significant, greatly limiting access in developing countries where submucous fibrosis is most prevalent. Of particular relevance to the
Trial of a Physiotherapeutic Strategy for the Management of OSF

current thesis is the use of stretching exercises in combination with surgery, raising the possibility that the exercises alone may have significant effect, although this was not tested in the reported study (Lai et al., 1995; Meghji and Warnakulasuriya, 1997).

5.1.2.iii Combination of surgery with physiotherapy in the management of oral submucous fibrosis

The combined use of surgery to relieve the fibrous tissue, with placement of tissue grafts, together with cessation of the areca chewing habit and regular exercises results in improvement over an extended period of time, especially in those subjects with significant trismus defined as an inter-incisal distance of less than 20mm (Lai et al., 1995). Supporting such approaches have been the application of oral splints in conjunction with surgery to reduce the rate of relapse (Le et al., 1996). However such splints appear to require constant and long term use, or else relapse is frequent. Unfortunately treatment regimes in the study were assigned according to the degree of trismus and this makes interpretation of the results difficult (Le et al., 1996).

5.1.3. Limitations of currently practiced methods for the management of oral submucous fibrosis

Intra-lesional injection of interferon gamma has resulted in promising improvement for patients and also provided some potential insight into the underlying disease mechanism (Haque et al., 1998). However, there are substantial limitations to this therapeutic approach. Importantly, a granulomatous response at the injection site provides worrying evidence for potentially destructive long-term side-effects (Walther and Hohlfeld, 1999). Equally important, is the expense of the intralesional
injection, requiring pharmaceutical agents, injection equipment and an appropriate health workforce, none of which are sufficiently widely available in many of the developing countries where OSF is prevalent.

A summary of the present options available for the management of OSF could be made as follows. Firstly, although direct pharmacological approaches for management of OSF may be more practical for delivery and do offer some respite from oral discomfort, the major symptom of trismus remains unrelieved. Secondly, with regard to the surgical strategy for management of OSF, not only does this short-term treatment almost invariably result in recurrent and worsening disease (Lai et al., 1995), but the surgical workforce and resources needed to manage an estimated two million sufferers is simply not available. Therefore, there is a pressing need for the development of an alternative approach to the management of OSF that is more consistent with the capacity of health care systems in developing countries.

5.1.4. An alternative conservative treatment approach to management of oral submucous fibrosis

Work described in Chapter 4 raised the possibility of an immunological basis for OSF, consistent with the response of this disease to Interferon gamma injection (Haque et al., 2001). If such an immune basis is assumed, then it seems reasonable to suggest that this response might be driven by some as yet unidentified, component of the areca nut. However, it is also possible that other substances could initiate a similar response in susceptible individuals.
Trial of a Physiotherapeutic Strategy for the Management of OSF

Even disregarding such a possible immune basis to OSF, on first principle the treatment of any condition usually involves removal of the causative agent. For this reason it is here argued that any conservative treatment of OSF must include cessation of the areca nut chewing habit.

Separately, it is possible that once an initial immune response is stimulated by a 'trigger' allergen, that agents other than the initial allergen may be able to propagate subsequent further responses despite the absence of the 'initial' trigger, perhaps even a common irritant like capsaicin. It is of note that many people with OSF report they do not chew the areca nut (Section 1.9.2.iii and 4.1.ii), while people with OSF respond strongly in extreme pain to capsaicin spice which is widely used throughout the world and yet OSF is only found in areas where the areca nut is chewed. It may be that even a brief and easily forgotten exposure to areca nut early in life predisposes susceptible people to development of OSF in response to other agents at a later time.

Trismus is a critically important sign of OSF and any form of management necessitates relief of this sign. In as much as OSF may be viewed as a disease of imbalanced extracellular matrix deposition and removal, the fact that the extracellular matrix is turned-over raises the possibility that even diseased tissues may be remodelled using a physiotherapeutic approach. Such a strategy not only exploits normal physiological mechanisms to overcome trismus, but can also be delivered in poorly resourced health systems.

The current Chapter describes a clinical study in which a physiotherapeutic approach was trialled. Recognizing the pain experienced by some patients during oral
movement, patients were advised to use non-steroidal anti-inflammatory analgesics as needed, while there was also advice to cease areca nut use.

5.2. Materials and Methods

5.2.1. Materials

Hyaluronidase and hydrocortisone were purchased from Sigma-Aldrich (Sigma-Aldrich Corp. Bangalor, India). Mebendazole and ferrous sulphate, measuring callipers, wooded tongue spatulas, syringes, and needles were purchased from Nepal Pharmaceuticals (Old Baneshwor, Kathmandu, Bagmati Nepal). Local anaesthetic comprising 2% lidocaine with 1/100,000 adrenaline was purchased from AstraZeneca India (AstraZeneca Bangalore, India).

5.2.2. Methods

5.2.2.i. Patient inclusion and evaluation

Patients presenting with subjective reduction in oral opening over a 5-year period to Patan Hospital in Kathmandu Nepal as described in Chapter 4 were included in this study. Ethical approval was from the Ethical Committee of Patan Hospital. As outlined in Chapter 4, trained investigators interviewed patients for gender, age, areca nut habit, and occurrence of pain; and examined patients for measurement of inter-incisal distance by vernier caliper, identified sites with OSF that were confirmed by biopsy and also noted oral vesicles and/or ulcers. Blood tests for anaemia were offered, but accepted by only 34 participants. An overview of the study is given in Figure 5.1. Although data was incomplete for some patients, meaningful statistical
evaluation by Wilcoxon’s Ranked sign, Mann-Whitney U, and Chi-square tests was possible.

Inter-incisal distance was determined in two recordings per visit between left maxillary and mandibular central incisors, or nearest appropriate teeth if absent. Measurements were always passive without force applied. The lower limit of normal inter-incisal distance in the Nepali population was recognized as 32 mm (Cox and Walker, 1997).

5.2.2.ii. Assignment of treatment group

Of 61 OSF patients presenting, 7 were excluded: 3 (2 females and 1 male) because of disease so severe that surgery appeared the only reasonable approach; and 4 (1 female, 3 males) for concomitant OSCC. Random numbers were used for assignation to control, injection with hyaluronidase and steroid, or physiotherapy of 54 subjects (38 male mean age 36.4, 16 female mean age 35.1). However, patients unable to attend bi-weekly injection were assigned for physiotherapy with the next subject assigned for injection. Baseline characteristics for the study are given in Table 5.1.

Control and injection enrolment ceased for ethical reasons when sufficient control patients returned and injection was recognized as having poor outcomes. Only 28 (52%) of initially enrolled patients returned for 4-month evaluation. The hyaluronidase and steroids injection group had the lowest return rate of just 4 people (27%). Sixteen (70 %) patients receiving physiotherapy completed the study, while 8 (50%) of the control patients completed. Figure 5.2 shows the distribution of patients
Figure 5.1

Study overview demonstrating the allocation of participants, numbers lost to follow-up, and numbers analysed.
## Overview of Trial

Participants
61

- 7 Excluded
  - 3 Severe disease
  - 4 Concomitant O/SICC
54 Subjects

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### Table 5.1 Data obtained from OSF patients using standard form (Figure 4.1)

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Fifty-four subjects participated in the study. Six subjects indicated that they had no smoking or chewing habit. Oral mucosal involvement ranged from (1) soft palate only to (6) where soft palate, pillar of the fauces, bilateral buccal mucosa, and labial mucosa were involved. The treatment groups were: 1 – control, 2 – Injections and 3 – physiotherapy.

Records 1, 13, 22 & 41 were removed due to concomitant OSCC, and 51, 57 & 61 due to severe disease requiring surgical intervention.

... into each of the three study groups with regard to oral opening and mucosal sites affected with reference to the data presented in Chapter 4 (Figure 4.4).

5.2.3. Treatment common to all three groups

All patients were advised of the association between areca nut and OSF and recommended to cease areca nut use and to assume a bland diet avoiding spicy foods. Twenty-four males and 10 females accepted blood tests and of these, 6 females (60%) and 7 males (29%) were anaemic so that anti-helminthic and haematinic supplements were prescribed comprising Mebendazol 100 mg twice daily for 3 days, ferrous sulphate 200 mg daily, and folic acid 5 mg daily. Two females and 2 male anaemic patients were in the control group; 2 females and 2 male anaemic patients in the hyaluronidase and steroid injection group; and 2 females with 3 male anaemic patients received physiotherapy. Treatment of all groups was for 4 months followed by evaluation with opportunity for monthly recall for support.

5.2.4. Management of patients in each group tested

The untreated control group comprised 13 males and 3 females with ages ranging from 19 to 77 years (average 35.8 ± 14.9 years).
Trial of a Physiotherapeutic Strategy for the Management of OSF

The group treated with hyaluronidase and steroid comprised 9 males and 6 females aged from 12 to 62 years (average 37.7 ± 16.1 years) and received bi-weekly submucosal injections over four weeks of hyaluronidase (1,500 units) and hydocortisone (100mg) in sterile water, with a total volume of 4 ml, as published elsewhere (Borle and Borle, 1991; Chaturvedi, 1989).

The physiotherapy group comprised 16 males and 7 females from 17 to 60 years (average age 35 ± 12.5 years). Patients were asked to undertake jaw exercises five times a day, in which tongue spatulas were positioned passively between anterior teeth and spatula number determined by comfortable maximal oral opening. Each session was for 5 minutes, 5 times a day. An additional spatula was added every fifth day unless this caused pain, in which case the additional spatula was added on the tenth day. Physiotherapy was to take place without pain, considered a sign of inflammation and potential exacerbation. To reduce discomfort, patients were encouraged to take aspirin (200mg) or paracetamol (250mg) 30 minutes prior to the exercise. Few participants took these analgesic agents, seemingly due to cost.
Figure 5.2

Scattergram showing oral opening according to the progressively increasing oral mucosal surfaces affected with OSF and indicating to which of the three treatment groups each patient was assigned. OSF affected the oral mucosa in a highly predictable way, such that fibrosis affected first the soft palate, and then the fauces, unilateral buccal mucosa, bilateral buccal mucosa, floor of mouth and lips in that order. This was accompanied by progressive reduction in the inter-incisal distance (p < 0.006), with most patients presenting with an oral opening less than 32 mm (dashed line) being the lower end of the normal range of opening in Nepal. There was a random distribution of patients with regard to assigned treatment group.
Progressive Involvement of Oral Mucosa

- Soft Palate
- Fauces
- Buccal Mucosa (Unilateral)
- Buccal Mucosa (Bilateral)
- Floor of Mouth
- Lip Mucosa

- No Treatment
- Hyal. & Steroid
- Physiotherapy

Inter-Incisal Distance (mm)

10 20 30 40 50 60
5.2.5. Statistical Evaluation of Results

Statistical evaluation was undertaken using the Wilcoxon’s Ranked sign test to evaluate changes within treatment groups, while the Mann-Whitney U test was used for comparison between groups. The Chi-square test was used to compare relative proportions. Throughout, p values of less than 0.05 were considered to indicate statistically significant differences.

5.3. Results

5.3.1. The major clinical symptoms of oral submucous fibrosis were restricted oral opening and pain

Since the patients included in this study were the same as described in Chapter 4, the clinical findings are essentially similar to those already described (Chapter 4). Nonetheless, the patients included in the current treatment study comprised a sub-set of those described in Chapter 4, so that it seems important to provide the specific clinical details of these participants.

More males (38, 70%) presented with OSF than females (16, 30%) (p < 0.05). Of the 48 participating OSF patients for whom areca nut habit was recorded, (32 males, 16 females), 4 patients (8.3%, 1 male, 3 females) denied areca nut use.

While all 54 OSF patients subjectively experienced reducing oral opening, 44 (81%) presented with an inter-incisal distance ≤ 32mm, the lower limit of normal opening in
Nepal. Twenty-one of 34 (47%) patients for whom a pain history was available reported oral mucosal pain. There was no statistically significant difference between the incidence of pain or reduced opening, while there was similarly no statistically significant difference between the sexes with regard to pain or opening. Nonetheless, 13 of these 34 patients (38%) reported pain during eating, while 3 out of 34 (9%) had almost constant pain. As noted in Chapter 4, there was no clear relationship between initial oral opening or the presence or severity of pain.

The presence or absence of vesicular or ulcerative oral lesions was recorded for only 27 of the OSF patients who were included in the current study, but of these, 11 (41%) presented with one or other of these lesions. Vesicles presented in 10 of 27 patients (37%), being 3 of 4 females (75%), and 7 of 23 males (30%). Ulceration was in 6 of 27 patients (22%), being 1 of 4 females (25%) and 5 of the 23 males (21%) recorded. The two types of lesions usually occurred together, such that only one of the patients with oral ulceration did not also have vesicles. Not all patients with vesicles or ulcers reported pain, with 5 (2 females, 3 males) out of 11 patients with vesicles or ulcers, not volunteering a history of pain. There was no statistically significant difference between the sexes with regard to the incidence of pain or distribution of vesicular or ulcerative lesions.

5.3.2. Oral opening improved with physiotherapy

Sufficient patients in both the physiotherapy and control groups returned at 4 months to permit a meaningful statistical evaluation of physiotherapy. There was no significant improvement amongst control patients, but there was substantial improvement in patients undergoing physiotherapy ($p < 0.0005$) (Fig. 5.3). Figure 5.4
shows the relationship between initial oral opening and the response to physiotherapy and it is of note that the only two patients who failed to improve with physiotherapy had started the study with inter-incisal distances in the normal range. There was no clear association between outcome with physiotherapy and any other parameters including gender, the presence and severity of pain, and the occurrence of vesicles or ulcerative lesions. Figure 5.3 also demonstrates an inverse association between the inter-incisal distance before physiotherapy and the change in oral opening after 4 months of physiotherapy. A correlation analysis of this confirmed this clinically significant finding with \( y = -0.3x \) \((R^2 = 0.129)\).

Oral pain after treatment was recorded for only 14 of the patients completing the study. However, amongst those for whom oral pain was recorded, there was no change for 10 patients. Improvement was seen in only 2 male patients, who had both received physiotherapy and improved from pain on eating in one case, or constant pain in the other. In contrast, 2 patients reported worse pain, progressing from that only on eating to being constant, while one of these was a female receiving hyaluronidase and steroid injections, and the other was a male receiving physiotherapy. The female who suffered this increase in pain with hyaluronidase and steroid injections also had a reduction in oral opening of 2mm. In contrast, the male who had increased pain with physiotherapy, enjoyed increased oral opening of 6mm. Data suggest that the physiotherapeutic strategy for treatment, although improving oral opening, did not affect oral pain.
Figure 5.3

Scattergram showing the change in inter-incisal distance after 4 months of either the absence of any active treatment, or alternatively treatment with hyaluronidase and steroid injections, or physiotherapy. There was no statistically significant change in oral opening in the group without active treatment and this contrasted strongly with an increase in oral opening for all but two patients who received physiotherapy (p < 0.0005). Insufficient patients receiving injections of hyaluronidase and steroids returned for evaluation to permit meaningful statistical evaluation of this mode of treatment.
Figure 5.4

Scattergram showing the relationship between the inter-incisal distance before physiotherapy and the change in oral opening after 4 months of physiotherapy. The two patients for whom physiotherapy did not improve oral opening commenced the study with inter-incisal distances in the normal range (over 32mm), so that improvement was seen in all patients who had objectively verifiable restriction in oral opening.

A correlation analysis demonstrated an inverse association between inter-incisal distance prior to physiotherapy and the change achieved after a period of 4 months with \( y = -0.3x \) and \( R^2 = 0.129 \).
5.4. Discussion.

Recognizing reduced inter-incisal distance as a primary sign of OSF, this measure was considered a reasonable objective indicator of disease status, while it was possible to relate inter-incisal measurements to an established Nepali population norm for minimum normal opening (Cox and Walker, 1997).

Most people with OSF in the current study used areca nut, supporting an association between areca nut use and the disease. However, a proportion of patients denied the habit, consistent with earlier observations (Seedat and van Wyk, 1988) and suggesting that areca nut may not be directly causal of OSF, but perhaps permissive of other unidentified factors. The higher incidence of OSF encountered in males compared with females in the current study, may reflect greater use of areca nut in this group.

The most important observation made in the current study was that physiotherapy substantially improved oral opening in patients with OSF. This was consistent with earlier reports where physiotherapy was combined with other treatments (Lai et al., 1995; Sharma et al., 1987). The current study seems unique in that physiotherapy was trialled as an independent treatment and was effective. However, it should be noted that there are single case reports of individual patients where a primarily physiotherapeutic strategy was effective (Reichart and Philipsen, 2006; Yusuf and Yong, 2002).

This study was performed over a period of 5 years, and involved a number of clinicians, and one effect of this was that progressively more data was collected from patients with regard to their pain experience and oral mucosal lesions as the study
progressed. Because of this, less data was available for eventual analysis of some aspects of the study, compared with others. Nonetheless, it was possible to make statistically meaningful statements about most of the more important questions addressed in the study, although it would clearly be desirable for any further work to take advantage of the current experience to establish more complete data sets.

It is unfortunate that there was a high attrition rate for participants, so that it was not possible to make statistically meaningful statements regarding the efficacy or otherwise of treatment with hyaluronidase and steroids. Nonetheless, the absence of clear improvement in those patients who did attend for completion of the study did not encourage this clinical strategy. It is possible that non-attendance by a majority of patients receiving injections was due to successful treatment. However, this seems unlikely in view of the reasons offered for non-attendance by those patients who did respond to inquiry. It is also noted that relatively small numbers of patients were involved, so that the apparent clustering of non-attendees in the group treated with hyaluronidase and corticosteroid may be circumstantial. A further possible interpretation is that having suffered repeated painful injections, patients felt unwilling to return for further assessment. While a significant number of patients attended for initial assessment, their desire for a quick cure was not fulfilled. Instead they were given advice about discontinuing an established habit, avoiding spicy foods that make eating more pleasurable, and a warning that this will have to continue for many months. This was frequently poorly received, and although the hyaluronidase and steroid therapy was initially popular, this soon changed when minimal improvement was seen by patients and discomfort increased.
Trial of a Physiotherapeutic Strategy for the Management of OSF

Despite these unavoidable limitations, the current study did demonstrate a substantial improvement in oral opening in patients treated by physiotherapy.

Limitations in the study design should also be declared. These would include the bias created by the large number of drop-outs from the study especially in relation to the ‘injection’ treatment option; the lack of any blinding in the study and the pseudo-randomisation method of allocation of subjects entering the different treatment regimes.

The possible contribution of non-steroidal analgesics to the effect of physiotherapy was difficult to evaluate, primarily because of poor compliance and also because the necessary data were not collected. However, it does seem unlikely that these drugs were important, not only because of low compliance, but also because aspirin and paracetamol were both recommended, and both have very different mechanisms of action. Also, these drugs are widely available and used, while there are no reports of successful management of OSF with either of these agents alone. Similarly the very small number of patients receiving anti-helminthic treatments and haematinic supplements, as well as the essentially equivalent distribution of these patients across treatment groups, makes it unlikely that these systemic treatments had any effect upon the observed results.

The lack of correlation between oral opening and pain was surprising, while failure of physiotherapy to impact on oral pain supports the idea that physiotherapy did not address the underlying basis for OSF, but only mediated its effect by facilitating advantageous tissue remodelling. Supporting the idea that physiotherapy improved oral opening by remodelling of the tissues, was that improvement in oral opening was
only unsuccessful in those patients who already had opening in the normal range, so that only negligible remodelling and improvement might be expected. It was also interesting that OSF progressed in a highly predictable manner, with buccal mucosa never affected in isolation to the soft palate and the anterior pillar of the fauces for example. As noted in Chapter 4, the current study appears the first report illustrating this highly predictable pattern of disease in Nepal.

With reference to the suggestion made in Chapter 4 that OSF may reflect a Type IV hypersensitivity response, while the percentage of the population that is sensitive to any putative nut allergen is probably small, this substance is nonetheless widely used throughout many Asian cultures so that individuals may come in contact with small quantities of areca nut without consciously knowing this to be the case.

Supporting the association between nut use and OSF is that the current study found most OSF sufferers used the areca nut intentionally, while as seen in Chapter 2, between 30% and 50% of the population report no history of areca nut chewing.

It would be desirable to expand the currently described study in future work, training health service personnel in regional areas to assess and manage the clinical symptoms of OSF and ensuring that patients do not have to travel large distances for reviews. This would hopefully increase the follow-up rate and also provide a larger and more complete data set for analysis. Multi-centres studies involving both inter-country as well as intra-country sites would also allow evaluation of different responses of patients in different areas with different patterns of areca nut use and OSF. With regard to this, it would be further interesting to obtain additional information on other
factors such as smoking, diet or systemic disease upon the efficacy or otherwise of the treatments trialled.

Finally, the failure of physiotherapy to address the problem of oral mucosal pain requires further work to develop a suitable strategy for this important and troubling symptom. It is possible that combination of physiotherapy with an effective pain relieving treatment may successfully alleviate the symptoms of OSF, despite what may be a continuing lack of understanding of the underlying biology of this interesting disease.
Chapter 6

General Discussion
6. General discussion

This work was aimed at understanding OSF with regard to its relationship with areca nut use, pre-malignancy and OSCC. Recognizing the importance of regional variation, the thesis focussed on the little studied Nepali population. To approach a better understanding of OSF, laboratory based studies investigated the intra-oral concentrations of arecoline suggested by others to be important in OSF (Harvey et al., 1986; Meghji et al., 1987; Meghji and Harris, 1995; Murti et al., 1995; van Wyk et al., 1995). Also, work identified novel histopathological features of the disease which may lead to improved understanding of OSF pathogenesis. Finally, recognizing the lack of suitable treatment regimes for OSF in communities where the disease is most prevalent, a simple physiotherapy approach was trialled which could be readily implemented in affected communities.

As outlined in Chapter 1, areca nut is widely used in Asia, and although its historical origins are in Malaya, areca nut is now grown in export quantities throughout most of Asia. Use is widespread, particularly in the subcontinental region and the Pacific basin countries. Although there is substantial literature detailing patterns of areca nut use and associated oral mucosal lesions in most of the larger communities affected (India, Pakistan, Sri Lanka, and Taiwan), there has been a particular paucity of knowledge of areca nut use in Nepal and this was addressed by the epidemiological study described in Chapter 2. Despite the limitations detailed in the discussion of Chapter 2, data collected provide a valuable base-line for the planning of both future public health initiatives in Nepal, as well as expanded epidemiological surveys of the Nepali population.
General Discussion

The epidemiological findings in Nepal were largely consistent with those reported elsewhere (Daftary et al., 1980). However, the absence of either additive or synergistic interaction between tobacco and areca nut use, with regard to the occurrence of pre-malignant oral mucosal lesions, was interpreted as suggesting a ‘promoter’ rather than ‘initiator’ carcinogenic role for areca nut. Tobacco has significant carcinogenic initiator activity by causing direct DNA damage (Lodovici et al., 2007), and has high carcinogenic promoter activity stimulating the proliferation and consequent expansion of clones of cells with potentially damaged DNA (Yim et al., 2007). The low but nonetheless significant background rate for pre-malignant oral mucosal lesions in people not using tobacco or areca nut (Chapter 2), confirms a background rate for carcinogenic initiation and data are consistent with the idea that areca nut habits promote the clonal proliferation of cells with these ‘background’ genetic injuries. It is argued that if areca nut had significant initiator activity, there would be an at least additive effect above that of tobacco for the development of pre-malignant oral lesions. It is further argued that initiated cells in tobacco users are already maximally stimulated to proliferate by tobacco’s promoter activity, so that areca nut is unable to drive any further cell proliferation in those with tobacco habits, despite the apparent promoter activity of areca nut alone.

The experience gained from work described in Chapter 2 will be valuable in designing a further and much larger study, of not only the Nepali population but also communities outside of Nepal where areca nut is used. Data would be enriched by collection of more detail regarding the highly variable individual methods of areca nut and tobacco use. It would also be interesting to further investigate the model of areca nut as a carcinogenic promoter rather than initiator, with direct laboratory
investigations using cultured cells as well as animals. The classical approach of painting putative initiating and promoting solutions in varying combinations and in different order onto the backs of mice could be readily modified, using polycyclic hydrocarbons as a classical initiator, and saliva collected during areca nut chewing as a putative promoter for comparison with crotan oil as a known promoter. Such studies could be further expanded looking at the effect of saliva through which tobacco smoke has been bubbled as an 'initiator - promoter', with and without application of polycyclic hydrocarbons and 'areca nut saliva'.

Areca nut habits have travelled across the globe, with the emigration of Asian and Pacific island communities. The small survey on the use of the areca nut in Australia is outlined in the Appendix (as it examines 'use' only and not mucosal pathology associated with the use). It confirms this important trend and demonstrates the need for further both the development of preventive public health strategies in Australia, as well as planning for an expected increase in oral mucosal disease. Such planning would, however, require further more detailed survey of the Australian population in order to properly distribute public health resources. The current study, however, does provide a base-line from which additional surveys can be built, and also demonstrates the need for such further work.

The interpretation of areca nut as a potential carcinogenic promoter has relevance with regard to observations made of arecoline concentrations in saliva in Chapter 3. Not only were arecoline levels comparable to those demonstrated by others as able to stimulate and / or injure cells in culture, but importantly data suggested that salivary recirculation of arecoline occurs after it is absorbed across the mucosal membrane.
General Discussion

This is different to the entero-salivary recirculation as it is normally understood. Such recirculation would substantially increase the potential carcinogenic promoter activity of arecoline, so that a further discrete study of possible salivary recirculation would seem important. Again, a direct promoter activity for purified arecoline could be readily demonstrated in animal experiments using the approach outlined above. Also, salivary recirculation of arecoline could be confirmed in animal as well as potentially human experiments, looking at the salivary secretion of arecoline administered subcutaneously, intravenously or via gastric tubes.

It was notable that as detailed in Chapter 4, a significant minority of people presenting with OSF in Nepal denied use of areca nut. This was consistent with similar reports by others (Gupta et al., 1998; Hazaré et al., 1998), but does demand reconsideration of the putative role of areca nut in development of the disease. The novel observation also in Chapter 4, that epithelial cells in OSF may undergo vacuolation and degeneration in a manner reminiscent of known T cell cytotoxicity in lichen planus (Sugerman et al., 2002) suggests a means of reconciling OSF in people denying areca nut use, with the otherwise strong association of areca nut with the disease. If immune mediated cytotoxicity were responsible for OSF, it is possible that it is initiated by an as yet unidentified environmental agent in susceptible communities, while areca nut might in some way exacerbate the disease. This could be via stimulating increased expression of critical surface antigens, providing an exogenous source of material which in some way combines with cells to mimic antigens which are the target of inappropriately stimulated clones of T cells, or perhaps simply-evoking release of cytokines which enhance the auto-immune response.
The possible role of areca nut as an indirect stimulant of OSF is consistent with the putative carcinogenic promoter activity discussed above, in that stimulation of epithelial cell proliferation would both increase the number of potential 'targets' for self-recognizing clones of T cells, while promoter activity would also increase expression of any proliferation associated putative self-antigens.

It would be interesting to pursue these possibilities in experiments characterizing the T cell populations in OSF, as well as expression of epithelial antigens. Strategies would employ both cell culture based experimentation of epithelium treated with areca nut extracts, with or without mixed lymphocyte cultures, as well as characterization of cytokine interactions between lymphocytes, epithelium and dendritic cells in response to areca nut material. There is already a substantial literature investigating similar questions (Chiang et al., 2002; Haque et al., 1997; Haque et al., 1998), while observations in this thesis support the potential value of further such studies, but perhaps more appropriately targeted towards lymphocyte mediated epithelial cell cytotoxicity. Improved characterization of T cell sub-populations, as well as of the epithelium in areca nut users with and without OSF would seem an important, particularly in light of the limitations of poor antigen preservation in material studied in Chapter 4.

The chapter on 'clinical and histopathological investigations' came after the study on 'arecoline levels in saliva', rather than before, because it was important to first determine that although the concentrations of arecoline were sufficiently high in the saliva to cause some of the pathology identified, it failed to adequately explain the
disease process overall. Only after establishing this could alternative aetiologies in the light of the histological presentation be discussed.

It seems likely that the immune mediated destruction of epithelium suggested by histopathological observations in Chapter 4, accounts for the greatly increased incidence of OSCC in patients with OSF, as epithelial cell destruction is reasonably expected to stimulate proliferation of surviving cells. In this way, OSF can be argued to have direct carcinogenic promoter activity, similar to other conditions where there is epithelial cell damage such as lichen planus, iron deficiency anaemia or dyskeratosis congenita (Axell, 1993; Handley and Ogden, 2006; Maresky et al., 1989; Mithani et al., 2007).

It is clear from the literature and data presented in Chapters 2 to 4, as well as from the discussion above, that the pathogenesis of OSF remains uncertain. Indeed, Tilakaratne et al (2005), in their review of aetiology and pathogenesis of OSF, identified six different mechanisms in the disease process and all were disconnected. Thus highlighting our present inability to understand the overall initiator in the disease process. Never-the-less these OSF patients require appropriate management and treatment, however most of these patients dwell in developing countries where access to appropriate health care services are frequently limited. Regardless of the role or otherwise of areca nut, as well as immune or non-immune mediated epithelial or fibroblast cell damage, proliferation or stimulation, there seems universal agreement that OSF involves active tissue remodelling. Recognizing this fundamental and universally accepted fact of the disease, a treatment strategy exploiting the tissue remodelling of OSF to drive tissues by physiotherapy into a more functionally useful
form was successful, as demonstrated in Chapter 5. Consistent with the philosophical basis for a physiotherapeutic approach, which can not address the immunological basis of the disease, but only exploits tissue remodelling for a better functional outcome, was that treatment did not improve oral pain (Chapter 5). However, the use of physiotherapy for the management of one of the main complaints is most appropriate in the setting of a developing country, for it has been shown to be effective and is not dependent upon expensive resources.

It would be interesting to pursue further work to better understand the basis for the pain suffered by patients with OSF, and the perineural lymphocytic infiltrates noted in Chapter 4 may provide a basis for this important and troubling symptom. Characterization of these perineural infiltrates might be informative, while separate studies characterizing expression of neuropeptides in OSF compared with controls might also prove valuable. Finally, it would be interesting to investigate mucosal levels of known pain eliciting agents such as bradykinin (Flores et al., 2001; Gibbs et al., 2007; Okuse, 2007).

Substantial further work is clearly required but is beyond the scope of the current thesis. Nonetheless, it is felt that the work described in this thesis provides a valuable basis from which further investigation may proceed.
Appendix

Areca nut use in Australia
A.1. Introduction

As people move to new countries, they are generally accompanied by their traditional habits. Studies in the United Kingdom show that up to 74% of South Asian children between 11 and 15 years of age use areca nut at least three times a week (Farrand et al., 2001). An adult population study of regular chewers in the UK demonstrated oral mucosal pathology in 25% of the subjects (Pearson et al., 2001).

Although this section does not sit logically with the argument of the overall thesis, for there is no data relating mucosal pathology with ‘use’ in Australia, nevertheless it is important data concerning the use of areca nut, and therefore should alert oral health physicians in Australia to the likelihood of mucosal pathology.

A.1.1. Areca nut use varies between different migrant communities in the United Kingdom

Chewing habits amongst Asians living overseas also show significant variations. Recently, three studies in the UK sought to determine the extent of the areca nut use amongst adults in that country (Pearson, 1994). These studies, which were focused on Bangladeshi emigrants, revealed a consistent high level of use ranging from 78% to 97%, while when Asians from other ethnic backgrounds were studied, lower levels were reported ranging from 27% to 47% (Atwal et al., 1996; Setty and Johnson, 1999; Vora et al., 2000). This suggests that different emigrant communities have different patterns of use, and this is potentially valuable information for health planners and clinical members of the health professions.
Unfortunately these studies did not appear to record the age profile of those who used the areca nut, so that it remained unresolved if use represented mainly the older members of the community who had perhaps maintained the habit commenced in their homeland. Neither do these studies address the question as to whether adolescent Asians living in these communities were using the areca nut. Some insight into these issues however, is provided by several separate studies of adolescent Asian migrants living in the UK, showing that from 22% to 77% of these young people engage in areca nut chewing (Farrand et al., 2001; Osman et al., 1997; Prabhu et al., 2001). Important for the present study, was substantial variability of the reported data between the studies, suggesting regional or cultural differences, or perhaps the ethnic background of these children.

A.1.2. The Asian population in Sydney demonstrates great diversity

Sydney is a multi-cultural city and has a substantial number of migrants from South and South-East Asia. The 2001 Australian census showed that 22% of the population was born overseas, and for the first time Chinese languages eclipsed Italian as the nation’s most commonly spoken non-English languages. More specifically, Sydney defined itself as the migrant centre of Australia, with 31% of its population born overseas, while Melbourne recorded 8% of its population as Asian-born. The census demonstrates that the Indian community is within the top 10 by birthplace, due partly to the large number of Indian fee-paying students attending colleges and universities in Australia. More than 10% of Australians now define themselves as being of Asian or middle-Eastern ancestry. With regard to the Indian sub-continent, the 2001 census data demonstrated 37,889 (0.6%) of people living in NSW were born in India, which is an increase of 32% above
that recorded for the 1996 census. The 2001 census also showed that 54% of the 28,000
Indians who spoke Hindi were born in Fiji.

While areca nut and chewing tobacco are not traditional in Australia, the multi-cultural
nature of the population in Sydney, as well as the large number of migrants from India
and South Asia, lead to questions if, and how much 'areca nut' is chewed in Australia.

A.1.3. A lack of evidence on areca nut availability and
use in Australia

Anecdotal evidence from many Asians living in Sydney is of ready access to areca nut
products and chewing within the metropolitan area. Hence, prior to work described in
this thesis, the candidate made initial inquiries regarding the availability or otherwise of
areca nut products suburban Sydney, and found areca nut products to be widely available
throughout Sydney and its suburbs. A clinical need to perform an appropriate survey was
supported by anecdotal reports of patients presenting to Sydney specialist clinics with
oral submucous fibrosis, as well as with oral cancer in combination with OSF.

Therefore, an important objective of the present study was to provide preliminary
information on the amount of areca nut available and used in an Australian setting. With
regard to any survey on areca nut use, it seems important to determine whether the nut is
used in the context of civil ceremonies as outlined in Section 1.4.d, or if use is primarily
to support personal chewing habits. For this reason the Australian survey described in
this chapter addressed the issue of the social context of nut use. Since Sydney is the
principal immigration centre for Australia, this initial survey focused upon this major city.

A.2. Materials and Methods

A.2.1. A targeted survey of areca nut use in Asian communities in an Australian population

A.2.1.i. Development of a questionnaire suitable for use in Australia

A modified form of the questionnaire first developed for use in Nepal was used in Sydney to determine if migrants from Asia practice their traditional chewing habits in Australia.

The questionnaire sought information on a standardized list of questions relating to age, sex, area code, country of birth, knowledge of chewing nuts, use and frequency of use of the nut, and finally when the habit had commenced for those who reported chewing.

The questionnaire was initially trialed with Asian migrants working at Westmead Hospital, a major teaching hospital in Sydney, Australia. As a result minor modifications were made to the survey process, such that members of the survey team took with them various samples of areca nut preparations, for purposes of clarification during interview.
A.2.1.i.1 Conduction of the Survey

Data was collected using the questionnaire on two separate occasions when large numbers of Asian migrants were gathered together for traditional festival events. The festivals that were chosen were the Indian Mella and Buddha’s birthday celebrations, on the basis that both these events were more specific for people from the Indian and South East Asian communities, rather than other community members. Also, these gatherings occurred in Sydney, permitting a broad sampling of the relevant immigrant community.

It was recognized that misunderstanding of the survey questions may result from language difficulties in communicating with some members of the migrant population. While the Hindi words for the areca nut have come across directly into the English language in the form of ‘paan and supari’, many of the words for areca nut products used in other Asian languages were not known. In an attempt to minimize this possibility, samples preparations of the areca nut were carried by the interviewers.

The large population of Asians living in Sydney, compared with other cities in the state of New South Wales, was an advantage for conducting the survey in Sydney. Supporting this was that the Detention Centre at Villawood, Sydney, encourages the development of a supportive community nearby, many of whom are Asians from South and Southeast Asia. With regard to the opportunity to trial the survey at Westmead Hospital, it was an advantage that there were many Asian migrants working in the health services at Westmead Hospital, while this suggested that large numbers of people with this background were present in the Sydney local communities. Also, numerous well attended Asian festivals were celebrated in Sydney, so that these festivals provided an opportunity to target the relevant group within the city.
A.2.1.ii.  Domicile of Australian respondents

The domicile of the respondents to the questionnaire was recorded as the Sydney postcodes. These regions were then divided according to their geographical distribution around Sydney into north, south, east and west, according to generally accepted geographical divisions within the Sydney community.

Despite grouping post-codes in this way, “Sydney West” was more difficult to define. In acknowledgement of widely accepted local convention, as well as to simplify the geographic distribution as much as possible, the following parameters were followed in defining “Sydney Areas” on the basis of postcodes: “West” comprised all regions west of the city of Parramatta; “South”, represented those suburbs south of the harbour and Parramatta river; “North” were those suburbs north of the harbour and Parramatta river; and “East” comprised the remaining suburbs East of Parramatta.

A.2.1.iii.  The survey form used in Sydney

The survey form used in Sydney is shown in Figure A.1. The survey sought to duplicate as much of the Nepal questionnaire as possible with regard to demographic data and use of areca nut.

A.2.2.  Data analysis

Odds ratios in the Nepali study were calculated by comparing the relative incidence of defined lesions in populations of males, females and both sexes together with defined oral habits, with the equivalent relative incidence of lesions in populations without any of the oral habits under study. In this way, an odds ratio of 1 indicates an equivalent
Appendix - Areca Nut Use in Australia

relative incidence of oral lesions between habit and non-habit populations, while an odds ratio of 2 would indicate double the relative incidence of oral lesions in the presence of the oral habit under study.

The statistical software packages SPSS Graduate Pack Version 13.0 for Mac OS X were used to analyse the data. The Chi-square test was used to evaluate the statistical significance of differences in proportion, while the Mann-Whitney U Test was also used where appropriate. Two-tailed tests were used throughout with statistical significance being accepted in instances where p < 0.05.
Figure A.1

Questionnaire used in the Sydney survey of areca nut use. The survey focused on areca nut habits, the age of commencement of the habits, and whether areca nut was used for exclusively ceremonial reasons. The incidence of use of traditional words or terms used to describe the areca nut was also determined in this survey.
# Survey of Areca Nut (betel nut) Use in Sydney

## General Information

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>........................... yrs</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>F / M</td>
</tr>
<tr>
<td><strong>Post Code:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Place of Birth</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Nationality (If not Australian)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Able to identify packet of areca nut</strong></td>
<td>Y / N</td>
</tr>
<tr>
<td><strong>Use of the areca nut</strong></td>
<td>Y / N</td>
</tr>
<tr>
<td><strong>Reasons for using the areca nut?</strong></td>
<td>Personal (oral) / Ceremonial</td>
</tr>
<tr>
<td><strong>If ‘oral’ what is the frequency of use?</strong></td>
<td>Daily □, Weekly □, Monthly □, Other ...............................</td>
</tr>
<tr>
<td><strong>Age when use first commenced?</strong></td>
<td>........................... yrs</td>
</tr>
<tr>
<td><strong>Do other members in the family use the areca nut?</strong></td>
<td>Y / N</td>
</tr>
<tr>
<td><strong>If ‘yes’ indicate the relationship</strong></td>
<td>Parent □, Grandparent □, Other ...............................</td>
</tr>
<tr>
<td><strong>Word traditionally given for the areca nut.</strong></td>
<td>Paan/Supari □, Betel □, Other ...............................</td>
</tr>
</tbody>
</table>
A.3. Results

A.3.1. Areca nut use in Sydney

A.3.1.i Demographic characteristics of participants in the Australian survey

Three hundred and thirty subjects volunteered to participate in the Australian questionnaire. Figure A.1 shows the age distribution of respondents. Amongst these, 145 respondents (44%) were female with a mean age of 38.2 ± 15.2 years, a minimum age of 14 years and a maximum age of 90 years. Male participants numbered 185 respondents (56%) and had a mean age of 40.1 ± 14.5 years, a minimum age of 9 years and a maximum age of 90 years.

Participants were from all parts of larger metropolitan Sydney, while the largest number of respondents 162 (49%) were from the southern region of Sydney, the highest prevalence of regular chewing was from the northern and western suburbs with 22.3% (27/121) of individuals. In total, there were 47, 162, 51, and 70 participants from the Eastern, Southern, Western and Northern suburbs respectively. Of the 59 chewers using areca nut on a daily, weekly or monthly basis, 5, 27, 11, and 16 were from the Eastern, Southern, Western and Northern suburbs respectively.
Appendix - Areca Nut Use in Australia

A.3.1.ii. People from the Indian subcontinent predominated amongst those surveyed in the Australian Asian community

The multicultural nature of the Sydney population was reflected by the large number of countries from which respondents came (Table A.1). Individuals who attended the Asian festivals in Sydney had diverse backgrounds with links to 29 different countries, excluding Australia. However, despite this diverse origin, approximately 40% of respondents reported coming from the Indian sub-continent, being India and Sri Lanka, with most Fijian participants also being of Indian extraction.

<table>
<thead>
<tr>
<th>Place of Birth</th>
<th>(n)</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia - 2nd generation</td>
<td>17</td>
<td>5.2</td>
</tr>
<tr>
<td>Africa countries</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>China</td>
<td>15</td>
<td>4.6</td>
</tr>
<tr>
<td>Fiji</td>
<td>33</td>
<td>10.0</td>
</tr>
<tr>
<td>HK</td>
<td>10</td>
<td>3.0</td>
</tr>
<tr>
<td>India</td>
<td>136</td>
<td>41.3</td>
</tr>
<tr>
<td>Indonesia</td>
<td>14</td>
<td>4.2</td>
</tr>
<tr>
<td>Laos</td>
<td>6</td>
<td>1.8</td>
</tr>
<tr>
<td>Malaysia</td>
<td>23</td>
<td>7.0</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>Taiwan</td>
<td>10</td>
<td>3.0</td>
</tr>
<tr>
<td>Vietnam</td>
<td>22</td>
<td>6.7</td>
</tr>
<tr>
<td>Other Asian Countries –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 countries including Pakistan, Burma etc.</td>
<td>25</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>330</td>
<td>100</td>
</tr>
</tbody>
</table>

Table listing the various countries of birth of the 330 respondents in the Sydney survey. The largest proportion of participants was from the subcontinent region (43%), with the second largest from Fiji, however the majority of these were again of Indian origin. The largest Asian group outside of the Indian sub-continent was Chinese (China, Hong Kong and Taiwan) with 35 respondents (10.6%).
Figure A.2

Histogram showing the age and sex distribution of respondents who participated in the Sydney areca nut chewing survey. There were 330 respondents, 145 females (white bar) and 185 males (black bar) and these were from a wide age range in both genders (9 – 90 years). The highest representation was in the 30-39 year age group.
A.3.1.iii. One third of ethnically Asian Australians chewed areca nut

Data collected on frequency of areca nut use in the surveyed Sydney population is summarized in Table A2, and illustrated in Figure A.3 In total, 33% of all participants reported use of the areca nut, while this was comparatively consistent across all age groups studied, ranging from the lowest relative proportion of 27.3% in people less than 19 years of age, and a maximum of 50% of people in the 60 to 69 year age group using the nut.

Of the surveyed population, 12.4% reported use of areca nut for ceremonial and religious reasons, while 20% chewed the nut regularly as an oral habit. Unfortunately, it was not possible to determine from the data set to what extent habitual chewers of the nut also used it for ceremonial reasons.

Overall, 17.6% of those surveyed chewed the areca nut as a regular habit, representing 14.5 % (21/145) of women, and .20 % (37/185) of males, while the apparently more regular use of areca nut by males was not statistically significant. Although there did appear to be a tendency for more regular use of areca nut use with increasing age (Figure A.3), this was not statistically significant. Nonetheless, the majority of people in the 30-39 and 40-49 year age groups reported regular over occasional use of the nut (p<0.03) (Figure A.3).
Figure A.3

Histogram showing the relative percentage of the Australian resident population surveyed using areca nut either occasionally (white bars) or at least monthly (black bars) according to age group, as well as the total number of respondents in each age group surveyed (n). Fifteen and a half percent of the population reported occasional use, while 17.6% of those surveyed reported use on a regular basis. The age group where areca nut use was most prevalent was that between 40 and 49 years, with 18.2% chewing areca nut on a regular basis. There was a general trend for the proportion of regular habitual chewers to increase with age while that of the occasional users diminished, although this was not statistically significant, while it was only in the 30-39 and 40-49 year age groups that more regular use of areca nut could be demonstrated as statistically significant over that of occasional use (p<0.03).
### Table A.2. The frequency of Areca nut use by Australian ethnically Asian migrants according to age group.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>(n)</th>
<th>Never</th>
<th>Daily</th>
<th>Weekly</th>
<th>Monthly</th>
<th>Occasionally (Less Than Monthly)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;19</td>
<td>22</td>
<td>16</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>20-29</td>
<td>66</td>
<td>47</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>30-39</td>
<td>92</td>
<td>63</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>40-49</td>
<td>74</td>
<td>48</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>50-59</td>
<td>41</td>
<td>27</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>60-69</td>
<td>20</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>70-79</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>80-89</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>90-99</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>330</strong></td>
<td><strong>221</strong></td>
<td><strong>17</strong></td>
<td><strong>22</strong></td>
<td><strong>19</strong></td>
<td><strong>51</strong></td>
</tr>
</tbody>
</table>

|          | **67.0%** | **5.2%** | **6.7%** | **5.8%** | **15.5%** |

The majority of Australian residents of Asian origin in Sydney who participated in the survey did not use the areca nut (67%). However, amongst those who did use the nut, 17.6% chewed the areca nut on a regular habitual basis defined as at least monthly, and 12% of the surveyed group chewed areca nut on a daily or weekly basis. There was no statistically significant difference in the pattern of use between age groups or genders.

#### A.3.1.iv. Areca nut chewing habits commenced at an early age amongst participants in the Australian survey, and was less prevalent amongst those born in Australia

Table A.3 shows the age at which respondents reported first use of the areca nut, with regard to the country of origin as well as use of areca nut more or less frequently than once per month. Eighteen percent of respondents used the areca nut at least once a month, while nut users born in the subcontinent were more likely to use the nut on a weekly basis (19.3%) compared with those born elsewhere 6.4%), (p < 0.001).

Twelve percent (3/26) of second generation migrant Australians used areca nut at least monthly. When subjects were grouped on the basis of ethnicity, 29.7% (43/145) of the
Indian participants used the areca nut on a monthly basis, which was significantly more than the 5.6% (6/107) of those from SE Asia (p < 0.001).

Eighty-one percent of those who chewed either daily or weekly commenced the habit during childhood or teenage years, with 73.7% of users reporting first use of the areca nut between the ages of 10 and 19 years, and 36% commencing prior to the age of 10 years. It is of interest that subjects who commenced chewing areca nut prior to 10 years of age were more likely to become ‘regular chewers’ (p < 0.05).

All Australian-born areca nut users commenced their habit below the age of 20 years, and it is important to note that relatively fewer Australian born respondents adopted areca nut use (11.6 %) compared with those born elsewhere (33.0 %) this difference was not statistically significant.

Similar to the experience in Nepal, there were some respondents (10/330) who were unable to recall when they commenced areca nut use, and this also applied to respondents who used areca nut at least monthly (4/59).

Although fewer females (28.3%) than males (39.5%) used areca nut (p < 0.04), amongst those using areca nut there was no clear difference between the sexes with regard to the age of commencement of use. Thirty-two percent (13/41) of the females, and 30.1% (22/73) males, who admitted to an areca nut chewing habit had commenced nut use by 10 years of age. The majority of both male (82%, 31/38) and female (62%, 13/21) nut users commenced the habit during childhood or teenage years.
Two ethnically Asian males born in Australia commenced the habit prior to 10 years of age, whereas none of the females surveyed commenced the habit if born in Australia.

Respondents who were born in India have the highest figures for use of the areca nut. 16.7% (6/36) of second generation migrant Australians used the areca nut on a regular basis. When subjects were grouped on the basis of ethnicity, two main groups emerged (184 Indian, and 106 SE Asian). 27.4% (51/184) of Indian migrants used the areca nut on a regular basis, compared to migrants from SE Asia who had a lower incidence of areca nut use 5.7% (6/106).

It was interesting that a further 3% of the respondents indicated that they used the nut for religious or ceremonial reasons only.
### Table A.3. The age of first use of areca nut use according to gender and place of birth.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Habit commencement</th>
<th>Birth place</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(% According to Country of Birth)</td>
<td>Non-Asian Countries (Australian &amp; Non-Australian Born)</td>
<td>India</td>
<td>South Asia</td>
</tr>
<tr>
<td>Female</td>
<td>No Habit / Occasional Use</td>
<td>(91%)</td>
<td>39</td>
<td>(75%)</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Prior to 10 yrs</td>
<td>(7%)</td>
<td>3</td>
<td>(5%)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10 – 19 yrs</td>
<td>(2%)</td>
<td>1</td>
<td>(7%)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>20 + yrs</td>
<td>(0%)</td>
<td>0</td>
<td>(9%)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>(0%)</td>
<td>0</td>
<td>(4%)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>(100%)</td>
<td>43</td>
<td>(100%)</td>
<td>57</td>
</tr>
<tr>
<td>Male</td>
<td>No Habit / Occasional Use</td>
<td>(83%)</td>
<td>29</td>
<td>(67%)</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Prior to 10 yrs</td>
<td>(9%)</td>
<td>3</td>
<td>(8%)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>10 – 19 yrs</td>
<td>(6%)</td>
<td>2</td>
<td>(18%)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>20 + yrs</td>
<td>(3%)</td>
<td>1</td>
<td>(6%)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>(0%)</td>
<td>0</td>
<td>(1%)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>(100%)</td>
<td>35</td>
<td>(100%)</td>
<td>88</td>
</tr>
</tbody>
</table>

The majority of regular users commenced their habit during their teenage years (53%) (p < 0.3), with approximately a third of all users commencing the habit before prior to ten years of age. Amongst those born outside of Australia, India had the largest representation and also had the largest percentage of regular areca nut users so that 17.2% of these individuals used the nut at least monthly, with an overall use of 32.4%. Although proportionately more males (25.9%) than females (14.5%) used the areca nut, irrespective of country of origin this was not statistically significant (p < 0.2). The age of areca nut chewing habit commencement did not differ in a statistically significant way between the sexes according to country of origin.
A.3.1.v. Words used by Australian respondents to refer to areca nut

When asked to identify different preparations of areca nut in terms of the “mother tongue”, a range of names for areca nut were offered as shown in Table 2.9. People from India had more words to describe the areca nut compared with people from other regions though this may only reflect the great geographic and ethnic variation of India. Nonetheless, a substantial proportion of people were unfamiliar with areca nut and did not know what it was when shown the material (24%), but this was primarily confined to people born in SE Asia and Australia, while most respondents born in India or Fiji recognized the nut preparations shown.
### Table A.4. Words used to refer to the areca nut.

<table>
<thead>
<tr>
<th>Word used for areca nut (Language)</th>
<th>Birth place</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outside Asia</td>
<td>India/Fiji</td>
</tr>
<tr>
<td>Betel / Areca Nut (English &amp; Indian)</td>
<td>9 (24%)</td>
<td>83 (45%)</td>
</tr>
<tr>
<td>Bin Lang (Chinese)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Betel with tobacco (Taiwanese)</td>
<td>2 (5)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Paan/ Supari (English &amp; Indian)</td>
<td>5 (14)</td>
<td>82 (44)</td>
</tr>
<tr>
<td>Pinang (Malaysian)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sirih (Indonesian)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Trau Cau (Vietnamese)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Did not recognise the 'areca nut'</td>
<td>21 (57)</td>
<td>16 (9)</td>
</tr>
</tbody>
</table>

**Total** 37 185 108 330

A range of terms was used to identify the areca nut among the respondents to the Sydney survey. The most popular term was in fact a group of English, or rather Indian (Hindi) words that have come into the English language. These were of ‘paan’, ‘supari’, ‘betel’ or ‘areca’ nut and these terms were used by 61% of the respondents. Proportionately, the largest group unable to recognize ‘areca nut’ products were born in Australia or SE Asia (p < 0.001).

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### A.4. Discussion

The use of a questionnaire to obtain preliminary data on the incidence of areca nut use in Australia seemed reasonable. The sample population, while small, was representative sample of migrants from countries where a tradition of areca nut use is established. Random sampling of people attending the religious and ceremonial celebrations associated with the Indian Mella Festival, and Buddha’s birthday seemed a reasonable targeted approach. While some visiting tourists were originally included in the
questionnaire, the number of such participants was very small and these data were excluded from subsequent analysis.

One difficulty in the targeted questionnaire approach used in Sydney, was that there may be an undetermined bias in the type of person that attends festivals where the study was conducted. Areca nut is frequently used as part of religious ceremonies, so that participants may be more exposed, and or have greater access to, the nut compared with people of a similar background but not attending religious festivals. It is accepted that the current Australian survey would not be able to identify such potential bias.

However, on balance a survey questionnaire targeting the section of society thought to use the areca nut seems a reasonable means of obtaining baseline data. It is acknowledged that current data has limitations, this does nonetheless provide clear evidence for the use of substantial quantities of areca nut in an Australian population, while it is important to note that this has not been previously documented.

The data obtained indicates that the areca nut is used by a reasonably large percentage of the migrant community. Use of the nut is not only for ceremonial reasons, but also for regular and personal reasons. The questionnaire allowed respondents to identify the reason for using the areca nut, should it only be ceremonial, and these were identified as 'occasional' users, chewing the nut less than 12 times per year.

While there was no significant gender difference in the Sydney survey with regard to use of the areca nut, this preliminary data indicates an ethnic difference, with the Indian communities having the greatest use of the nut relative to other communities.
Appendix - Areca Nut Use in Australia

The areca nut is easily available in Australia, while many respondents indicated that they consumed the nut regularly. It was interesting that the traditional and colloquial names for areca nut were retained in within the communities sampled.

Comparable data concerning the use of areca nut amongst Asians living in non-Asian countries is available from the United Kingdom, where 27-47% of the relevant asian communities chew the nut (Atwal et al., 1996; Setty and Johnson, 1999; Vora et al., 2000). This was comparable with the current survey, where 19.3% of Asian migrants reported a regular areca nut chewing habit.

Although the adolescent population was not specifically examined in this study, and the number of such participants was small, 22.7% of the adolescent population indicated that they used the nut on a regular basis, this being either monthly or weekly. This was again comparable to observations in the United Kingdom, where 22% of adolescents in a mixed Asian community regularly used the areca nut (Osman et al., 1997).

An interesting, but perhaps minor, observation was that there were two age groups, 20-29 yrs and 40-49yrs, where females used the areca nut more than males. The reasons for this are unclear, however, this may reflect the fact that chewing areca nut is more socially acceptable than smoking in these age groups. While the survey form did not allow opportunity to explore the reason for the differences, further study in this area should include questions to probe this issue.

Results from this Australian survey seem important for the providers of oral health services in Australia. Some populations using the areca nut demonstrate a prevalence of
relevant oral mucosal lesions in 25% of subjects (Pearson et al., 2001), consistent with the current Nepali data. Since areca nut is an independent risk factor for oral cancer (Warnakulasuriya et al., 2002), it seems important for Australian health authorities to be aware of the significant use of this agent by many people in migrant communities.

This survey did not specifically enquire as to whether the areca nut was used with tobacco, though some respondents volunteered the information that tobacco was used with the areca nut. Data available from the UK indicate a 27-47% prevalence of tobacco use amongst a mixed Asian population (Atwal et al., 1996; Setty and Johnson, 1999; Vora et al., 2000). However, as each geographical area appears to be distinctive with regard to the prevalence of areca nut use, it is difficult to be confident in making direct comparison.

Both areca nut and chewing tobacco were found to be easily obtained in Sydney, while it is interesting to note that retail of chewing tobacco is illegal in Australia (Winstanley et al., 1995), it can be imported for personal use (Sachdev and Chapman, 2005). It would be interesting for further research to focus on the sales people in the Asian ‘spice shops’, and their awareness or understanding of the link between areca nut, smokeless tobacco use, and cancer.

Australia has recently introduced legislation requiring all cigarette and tobacco packaging to have both verbal and visual warnings on the outside of packets. It seems correspondingly important to gain information on the presence of chewing tobacco use in pre-packaged commercial forms, both with and without areca nut.
Regarding the domicile of participants, there seemed no clear geographic focus in Sydney for areca nut use. From the perspective of health service providers, this suggests that all areas in Sydney are at risk of areca nut habits, and therefore, that all clinicians should be made aware that their Asian patients may be engaging in high risk practices for OSCC and OSF. Appropriate targeted screening and follow-up for relevant oral lesions seems sensible.

While the number of participants in the Australian study was of necessity limited, the observations made could be confirmed and strengthened in future studies by liaising with the relevant health departments to collect appropriate data relating oral cancer and risk habits. Such data should allow use of areca nut and chewing tobacco to be more clearly linked with data on the prevalence of oral mucosal lesions.

It would be interesting to extend the Australian study to investigate oral habits in indigenous Australians, while there is already some data available regarding tobacco use and a high incidence of oral cancer in this group (Ivers, 2001; Subramaniam et al., 2005). There is a history of traditional use of similar agents by Australian aboriginals, in that a native plant, Duboisia hopwoodii, grows in central Australia and is the source of a nicotine product, pituri, which was and may continue to be used by Australian Aborigines (Watson, 2006). There does not appear to be any research into whether this local source of nicotine is still harvested and or chewed, and whether there is a link with the high incidence of oral cancer amongst Australian aboriginals (Subramaniam et al., 2005). It would seem important to investigate the use of psychoactive alkaloids amongst the Australian indigenous community, as well as amongst Australians of Asian origin.
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