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Oxidative stress, nutrition and neutrogenomics in periodontal health and disease

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My sincere thanks to Philips Oral Healthcare for their kind invitation to speak at this 2006 Emerging Trends Symposium. I'd like to start my presentation by saying a little bit about the historical data that investigated putative links between nutrition and chronic periodontitis. I shall take a slightly critical view before bringing you up to date with data from some more contemporary studies. After this, I'd like to say something about the biology and the science that underpins micronutrition and the way in which antioxidant micronutrients influence the way the cells of our body communicate with each other. This involves oxidative stress, free radical and antioxidant biology. I'll follow this by providing you with some of the evidence for systemic and local 'hyper-inflammation' underpinning the pathogenesis of periodontitis; the hyper-inflammation specifically being related to excess oxygen radical production. I'd also like to share with you some data from our own group on antioxidant depletion in chronic periodontitis, before finishing off by saying a little bit about the way in which micronutrients can affect the way cells talk to each other at the molecular level, with a view to giving you some insight into where future host-modulating therapies may develop on the back of our increased understanding of this complex area.

The very early studies of the 1960s, 1970s and 1980s didn't really provide us with any convincing evidence for nutrition as a risk factor for periodontitis. There were many reasons for this. Many of the studies at the time relied upon dietary questionnaires as a way of assessing nutrition, rather than serum biochemistry, and of course we know that what we eat isn't necessarily what we absorb, which in turn is not necessarily what enters our tissues. Today, serum biochemistry is the way that we tend to assess these parameters. In addition, modern questionnaires have been fairly widely validated in large epide-

miological studies. This study from Bob Genco's group really started the ball rolling again in nutritional analysis and periodontitis (International Association of Dental Research (IADR) 2003). The study analysed data from the National Health and Nutrition Examination Survey (III) (NHANES III) of the USA, and demonstrated that chronic periodontitis was associated with increases in age but also with a decrease in dietary vitamin C intake.

Unfortunately, the National Diet and Nutrition Surveys (NDNS) of the UK don't collect data on periodontal outcomes, but in their analysis of the NDNS data, Sheiham and Steele (1) and Sheiham *et al.* (2) demonstrated lower plasma retinol, α-tocopherol and vitamin C levels in edentulous patients (>65 years) relative to their dentate counterparts. This study in 2003 (3) based on another analysis of NHANES III showed significantly increased levels of β-carotene and vitamin C in the serum of dentate individuals than in those who wore dentures. Grossi *et al.* (4) presented data from another NHANES III analysis at the Hawaii IADR (2004), demonstrating decreased serum vitamin C levels in periodontitis subjects who smoked. They also showed some interesting intervention data that demonstrated improved periodontal outcomes in terms of attachment gain and pocket depth reduction when a chewable vitamin C supplement was utilized as part of non-surgical therapy.

I did some work last year with my good friend and colleague Thomas Dietrich (5) in Boston, and we presented this data at last year's IADR (Baltimore, 2005). This was an analysis of NHANES III, which utilized serum micronutrient biochemistry, rather than self-report by dietary questionnaire. We reviewed data from over 11 500 subjects and used multiple logistic regression to analyse associations between individual serum antioxidant concentrations and chronic periodontitis, adjusting for the confounders that we all know act as modifying factors for periodontitis. We assessed the following serum antioxidants biochemically; α- and β-carotene, selenium, lutein, uric acid, β-cryptoxanthine and vitamin C. We also used an algorithm which we had previously validated (6) that enables us to derive a measure of total antioxidant defence (TAOC) from the individual antioxidant components with 86% accuracy. We set up a number of mathematical models, correcting for various co-variates such as smoking, and then in line with recommendations of Hujoel *et al.* (7), we performed separate analyses that were restricted to never smokers.

Using a model which adjusted for age, gender, race and ethnicity, we found that virtually all the antioxidant micronutrients afforded significant protection, in that as serum levels increased the relative risk for periodontitis decreased. This also held true for total antioxidant capacity (TAOC), but in a model that added in adjustments for cigarette smoking, oestrogen use, diabetes and poverty–income ratio, we found that only β-carotene, selenium, vitamin C and bilirubin remained protective. In other words, as we saw a one standard deviation increase in the serum concentrations of these components, we found that the odds ratios for periodontitis decreased.

In the analysis of never smokers, we found that the protective effect still remained for vitamin C.

So to very crudely summarize what I've said so far, the early studies where nutrition was assessed by dietary questionnaire didn't really demonstrate any significant association with risk for periodontitis, whereas studies that have utilized serum biochemistry as the outcome measure of nutritional status do provide evidence that certain antioxidant micronutrients appear to afford some protection against periodontitis, particularly in smokers. There is also some evidence in never smokers, and in non-smokers, of a negative relationship between serum vitamin C concentrations and the relative risk of chronic periodontitis.

So what is the biological basis for this? What is the science that underpins this, if you like? One of the ways in which micronutrients can affect inflammation is through *oxidative stress*. Barry Haliwell and John Gutteridge defined oxidative stress very aptly as 'a condition that arises when there is a serious imbalance between the levels of free radicals in a cell and the antioxidant defence systems, in favour of the free radicals'.

Free radicals are reactive species capable of independent existence that contain unpaired electrons in their outer orbital, or shell, and those unpaired electrons confer upon free radicals a high degree of reactivity. The free radicals are constantly trying to extract electrons from other biomolecules in order to pair up their outermost electron, thereby achieving a structure which, according to the laws of thermodynamics, creates a lower energy state. The removal of the electrons is called oxidation and it results in damage to the oxidized biomolecules, following the free radical attack.

In recent years, the term *reactive oxygen species* (ROS) has replaced the term free radicals, as it's a more global term that includes species such as hydrogen peroxide and hypochlorous acid, which are not true radicals but nevertheless are capable of forming true radicals in the intracellular environment.

Antioxidants are biology's counterfoil to free radicals; they are substrates that will protect other substrates, even when they are present at lower concentrations, from oxidation. Like most things in life, redox homeostasis is about a balance between, on the one side the ROS, and on the other the antioxidants. If that balance shifts even just a small degree towards the ROS, this creates a low level of oxidative stress. Recent research has shown that such small shifts in the redox

state of a cell can activate gene transcription factors which are sensitive to such shifts, and these gene transcription factors, for example, nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), will stimulate pro-inflammatory cytokine synthesis and production, leading to indirect tissue damage through excess inflammation. If the redox state shifts in favour of antioxidants, then an anti-inflammatory state is created. Certain antioxidants will inhibit these gene transcription factors and downregulate and even switch off inflammation. Larger shifts in favour of ROS activity will create larger degrees of oxidative stress and induce direct tissue damage through mechanisms that I shall explain shortly, and larger shifts in the antioxidant direction will create an antioxidant state and activate DNA repair mechanisms.

So what are the biological effects of oxidative stress? They're generally grouped into two main categories:

- direct tissue damage;
- indirect tissue damage.

Direct tissue damage

There are many different methods by which free radicals can effect direct tissue damage. I will discuss the most widely understood and best described mechanisms, and I suppose the most relevant to periodontitis.

Lipid peroxidation is a chain reaction where a free radical attacks the cell membrane and sets off a series of radical chain reactions within the cell membrane, damaging it and ultimately causing cell death. However, some of the products of lipid peroxidation, such as the isoprostanes, are in fact pro-inflammatory themselves, and they can also be used as biomarkers of oxidative stress.

Protein oxidation: when free radicals attack proteins, they form carbon-centred radicals, and when carbon-centred radicals form next to each other, they can link to form a covalent bond, thereby causing a fold in the protein molecule and affecting the manner in which the protein functions. The stable products of protein damage are carbonyl groups, and again these can be measured as biomarkers of oxidative stress.

DNA damage by free radicals can result in strand breaks and base hydroxylations, changes thought to underpin what we now recognize as malignant change, as well as inflammation. 8-Hydroxydeoxyguanosine (8-OHdG) is a relatively stable biomarker of DNA damage and is now used as a measure of DNA damage through oxidative stress.

The *inactivation of protease inhibitors* such as α-1-antitrypsin is particularly relevant to us in periodontitis. This is the main inhibitor of neutrophil elastase and so when free radicals inactivate this molecule, you can see how indirect tissue damage results from the activity of elastase.

Indirect tissue damage

Indirect tissue damage is mediated via the activation of redox-sensitive gene transcription factors. The best characterized are NF-κB and AP-1, but there are a variety of others, and when they're switched on through oxygen radical activity, they produce a variety of pro-inflammatory cytokines and some anti-inflammatory cytokines. However, the overall effect is one of creating a pro-inflammatory and tissue destructive state.

Reactive oxygen species and peripheral inflammation

I want to say a little bit about peripheral inflammation caused through the generation ROS within the peripheral circulation. When neutrophils are activated, they contain a battery of enzymes and oxygen radical generating systems, primarily designed to destroy bacteria. When those systems are activated within the safe confines of the neutrophil membrane, then they're very effective. Ultimately, the neutrophils undergo apoptosis, and the relatively innocuous end points of apoptosis are removed by macrophages. Problems arise when the oxygen radicals are released into the extracellular environment, when host tissue damage results, which we now associate with periodontitis. There's been a lot of data in recent years, particularly from the Karolinska group and from Anders Gustaffson's team, looking at what they term neutrophil hyper-responsivity. These studies essentially involve collecting peripheral blood neutrophils from periodontitis patients and healthy controls and measuring oxygen radical production following neutrophil stimulation.

Periodontal bacteria produce and release into the bloodstream a variety of molecules that both prime and stimulate neutrophils: things such as f-mot-lev-phe (FMLP), bacterial DNA, and the one that we know most about, lipopolysaccharide (LPS). These agents at low concentrations will prime neutrophils, creating a state of readiness, for neutrophil activation. There are different ways of activating neutrophils, one common mechanism would be through opsonized bacteria and Fcγ-receptor stimulation, but toll-like receptor-9 (TLR-9) can also be stimulated directly by bacteria. Upon stimulation, the neutrophils take on board oxygen and they undertake what we call the 'respiratory burst'. The purpose of the respiratory burst is to produce the superoxide radical. This is a relatively benign oxygen radical, which doesn't cause a lot of damage, but it can

dismutate to hydrogen peroxide. Hydrogen peroxide is a sinister ROS when generated within cells, because it is capable of diffusion across cell and nuclear membranes, where it can sit next to DNA and other important biomolecules. In the presence of divalent iron and copper, it undergoes what we call Fenton reactions to form the hydroxyl radical, and it is this radical that is the most destructive radical we know of in human biology. Alternatively, myeloperoxidase can convert the hydrogen peroxide to hypochlorous acid and then hypochlorous acid will go on to produce a variety of other toxic molecules, such as chloramines and cytotoxic aldehydes.

ROS release from peripheral blood neutrophils

A couple of years ago we set out, in a Medical Research Council (MRC)-funded study to extend the excellent work of the Karolinska Institute, by examining the activity of unstimulated peripheral blood neutrophils in periodontitis patients and controls. This had not been possible before, due to limitations of assay sensitivity. However, we wanted to know whether the unstimulated neutrophils were also producing more oxygen radicals in their native and relaxed state. We did not really know what the effects of non-surgical therapy were on that activity state and so, in addition, we wanted to determine whether the reported hyperactivity was downregulated following successful non-surgical periodontal treatment. So, in this case–control study, we assessed differential gene expression profiles in the peripheral blood neutrophils from patients with periodontitis and age- and sex-matched healthy controls, all of whom were non-smokers, and we took 20 of each. We collected blood samples and prepared the neutrophils and then primed the neutrophils with different priming agents: LPS, granulocyte–macrophage colony-stimulating factor (GM-CSF), whole killed bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*). We employed unprimed controls. We then stimulated the neutrophils in different ways: Fcγ-receptor stimulation and TLR-9 stimulation with *F. nucleatum*. We also had unstimulated and unprimed controls. Total oxygen radical production was determined using the substrate luminol and we used isoluminol to determine extracellular radical release. This molecule is too big to cross the cell membrane and so when it is oxidized, it produces light, but it will only produce light if the radicals have been released outside the cell membrane. We then extracted the mRNA from the neutrophils and looked to see which genes were up- or downregulated in those cells. We used Affymetrix (Affymetrix UK Ltd, High Wycombe, UK) U133A arrays, which have about 15 000 genes imprinted on them, many of which are associated with inflammation.

When we looked at the light output from the luminol studies, we found the same as the Karolinska group and several other groups, in that we had significantly more total radical production in the patients at baseline (pre-non-surgical therapy) than the controls. Interestingly, when we treated those patients by traditional non-surgical means and then repeated these measures, we found that the total oxygen radical production significantly reduced to control subject levels. This indicated that the excess neutrophil radical production appeared to result from the periodontal inflammation rather than predisposing to that inflammation. However, when we then looked at extracellular radical production, and in particular ROS release from unprimed and unstimulated neutrophils, we found the same thing, i.e. we found excess radical release (albeit at a lower magnitude to the stimulated cells), from the patient's neutrophils relative to the controls. More importantly, when we treated those patients successfully, we found no change in the extracellular radical production, which remained significantly higher than the controls.

The gene expression data are complex and I just want to show you one table (Table 1), for one ontological group of genes, the interferon-stimulated genes. We found a large number of interferon-responsive genes upregulated by 5-, 5.7- and 4.63-fold in patient's neutrophils relative to the controls. These included type 1 interferons as well as type 2. Type 1 interferons as you know are produced in response to viruses, something that gives further strength to Jorgen Slots theories about a role for viruses in the pathogenesis of periodontitis.

We concluded overall that peripheral blood neutrophils from periodontitis patients who were non-smokers were both hyper-

Table 1. **Upregulated interferon-responsive genes in the hyperactive peripheral blood neutrophils of periodontitis patients relative to controls**

Accession number	Interferon stimulated genes	Fold change
A1337069	Viperin/cig5	5.9
NM_001548.1	IFIT	5.7
NM_005101.1	G1P2/ISG1	4.6
NM_006417.1	IFI4	3.7
BE888744	IFT	3.5
NM_001549.1	IFIT	3
NM_002462.1	MX1	2.9
NM_012420.1	RI58/IFIT	2.7
NM_015474.1	MG11	2.7
NM_003810.1	TNFSF1	2.5
NM_022873.1	G1P3	2.4
NM_021105	Phospholipid scramblase 1	2.4
AA083478	STAF50/TRIM2	2.3
NM_002759.1	PRKR	2.3
NM_017523.1	XIAP-associated factor-1	2.1
AF063612.1	OAS	2

active and hyper-reactive. The hyper-reactivity was decreased by successful periodontal therapy, but the hyperactivity seen in the unstimulated neutrophils was unaffected by treatment. The peripheral neutrophils also appeared to display a distinct molecular phenotype relative to non-periodontitis patients, and taken together overall, the data suggested the involvement of a peripheral stimulus, possibly a viral stimulus, possibly bacterial DNA, as well as a constitutional element to this neutrophil hyperactivity in periodontitis.

Evidence for ROS-mediated periodontal tissue damage

What evidence do we have for oxidative stress local to the periodontal tissues? Two studies in 2002 and 2003 both demonstrated increases in 8-OHdG levels in the saliva of periodontitis patients, and those levels decreased with treatment (8, 9). Sculley and Langley-Evans (10) in a cohort study showed increased levels of protein carbonyls in the saliva of periodontitis subjects relative to controls. Panjamurthy *et al.* (11) showed increased lipid peroxidation in both plasma and gingival tissues in periodontitis patients relative to controls. The problem we have at the moment is that there have been more review papers than original pieces of research in this area, and we really need some more original research.

Conclusions about ROS and periodontitis

Overall and taking all that information together, we can conclude that peripheral and local ROS activity in chronic periodontitis is significantly higher than in non-periodontitis subjects.

Antioxidant defence systems

So what about the antioxidant defence systems? A lot of the evidence to date has focused on peripheral antioxidant levels in serum. A study published in the *Journal of Clinical Periodontology* last year (12) demonstrated that serum vitamin C levels were inversely related to attachment loss in an elderly Japanese population. We know that vitamin C intake from the NHANES III data is reduced in periodontitis subjects relative to controls, and we know from plasma and biopsy data that the antioxidant scavenging systems are compromised in chronic periodontitis. The epidemiological data that Thomas Dietrich and I (5) presented at last year's IADR (Baltimore 2005) showed that the relative risk for periodontitis reduces as serum vitamin C levels increase, even in never smokers. However, there is a problem with all these data; most people focus on looking at individual antioxidants and their relationship to disease, but antioxidants don't work in isolation, they work in concert. The lipid peroxidation reaction is a classic example of how this takes place.

When a free radical attacks a cell membrane it sets up a lipid peroxidation chain reaction producing lipid peroxyl radicals, which damage and eventually may destroy the cell. Vitamin E is the most important lipid-soluble antioxidant we know of in human biology. It sits in the cell membrane, where it removes these radicals as they form, but in doing so, it is oxidized to the vitamin E radical. Regeneration of vitamin E from its radical is brought about by other antioxidants: in the lipid phase by ubiquinol and in the aqueous phase by ascorbate, and by an important molecule reduced glutathione (GSH). GSH is widely regarded as the most important extracellular scavenging antioxidant in the human body, and the reason for that is that when glutathione removes a radical, it is oxidized to a non-radical species (GSSG), whereas vitamin C isn't and vitamin E isn't; they form secondary radicals. So, GSH is a 'chain breaking' antioxidant, as it breaks the chain reaction.

So, if antioxidants work together, it is important to look at that global activity as well as looking at individual antioxidants. For this reason, a number of groups in the late 1980s and 1990s started to develop assays of TAOC. We developed one such assay in 1997 (13), an enhanced chemiluminescent assay, which utilizes a very simple concept. One simply puts luminol into a cuvette and then oxidizes it by adding hydrogen peroxide. The catalyst for this reaction is horseradish peroxidase, and the reaction produces a light signal. The light signal is totally dependent upon the production of luminol and enhancer radicals within the cuvette. If an antioxidant-containing solution is then added to that system [e.g. serum, saliva, gingival crevicular fluid (GCF) or homogenized tissue], then the antioxidants will scavenge the radicals and will switch off the light production until the antioxidants become exhausted, the radical activity restarts and light production returns. By measuring the delay from the switch off to the switch on of light production, we have our measure of TAOC. The assay is calibrated using a water-soluble vitamin-E analogue.

We applied this system in a case–control study published in the *Journal of Clinical Periodontology* (14). We looked at periodontitis subjects and age/sex-matched controls, all non-smokers. What we found was that in every fluid sample we looked at (serum, plasma, saliva, GCF), the TAOC of the controls was higher than the periodontitis subjects. This reached significance for plasma and was highly significant for GCF, but it didn't quite reach significance for serum or saliva. Other

groups have since done similar work and I think that if you summarize the literature now, the evidence indicates that peripheral and local antioxidant defence systems in periodontitis appear to be significantly reduced relative to non-periodontitis subjects. However, the big question is, whether this is a result of the periodontal inflammation (radical production during periodontal inflammation) or is it constitutional and predisposing to that inflammation? In order to try and answer that question, we took the patients from that original study and treated them using standard non-surgical therapy. We then remeasured their TAOC 3 months post-therapy. Pretreatment cervicular fluid TAOC in periodontitis patients was significantly lower than controls, and following treatment we saw a significant increase in TAOC. However, post-therapy TAOC did not reach the level of control subjects. Thus, the evidence points towards both total antioxidant compromise predisposing to periodontitis, but there is also evidence that part of the reduced TAOC may also result from the inflammatory process itself.

Antioxidants and cell signalling – the role of glutathione

How does all this relate to micronutrition and the way that cells communicate with each other? NF-κB is a redox-sensitive transcription factor which sits in the cytoplasm of cells bound to its inhibitor I-κB. When that complex is phosphorylated, the NF-κB is released from its inhibitor and is small enough to cross the nuclear membrane, bind to DNA and then to stimulate transcription of pro-inflammatory cytokines. It won't surprise you to know that the things which trigger that process via cell surface receptor binding are things such as LPS, interleukin (IL)-1, tumour necrosis factor-α (TNF-α) and reactive oxygen species such as hydrogen peroxide. What is interesting is that the process can be downregulated using the natural tripeptide reduced glutathione (GSH). We therefore looked at glutathione levels in our patients with periodontitis and the matched controls, prior to treating them and then looking again at the end of treatment. We found that GSH levels pretreatment in periodontitis were significantly lower in crevicular fluid than they were in the control patients, and treatment did not affect this difference, i.e. we were unable to improve GCF GSH levels with non-surgical treatment. What was even more interesting was the concentrations of GSH that we were getting in GCF were in the millimolar range, very similar to the levels found in the alveolar lining fluid of the lungs and about 1000 times higher than plasma levels. If you think about the lungs and compare their environment with that of the gingival crevice, they are in fact anatomically very similar. In lungs, there is an epithelial lining bathed by a fluid (alveolar lining fluid), and then beneath the epithelial lining are the connective tissues which experience intense neutrophilic inflammation in response to airway bacteria and other toxins. The situation is very similar to the sulcular epithelium and gingival crevice.

So, why is glutathione important? All this work led us to the hypothesize that in fact glutathione forms part of an innate defence strategy, not just within the lungs but in the gingival crevice. The reason we believe it is important is because:

- It is obviously a key radical scavenger.
- It is a chain breaking antioxidant.
- It is capable of downregulating and actually switching off inflammation when intracellular levels are boosted *in vitro*.
- GCF levels are 1000-fold higher than levels in plasma
- In disease, GSH levels in GCF are depleted. Why is it depleted in disease? We don't yet have any answer to that question, but it does raise another question – will increasing GSH levels inside cells be protective against chronic hyperinflammatory conditions such as periodontitis?

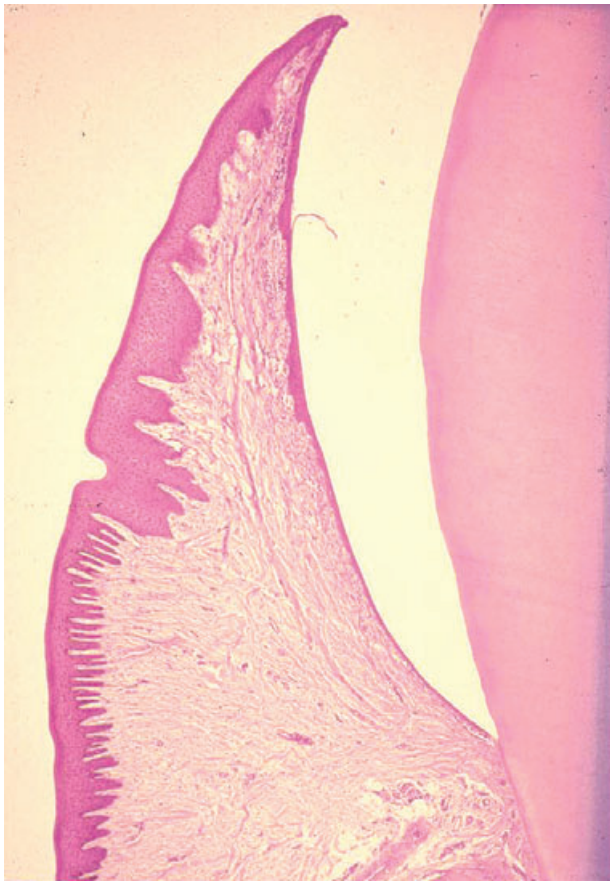

Fig. 1. The dento-gingival complex in health.

I'd like to give you a hint as to where we may be going with this. Figure 1 shows you the sulcular epithelium and junctional epithelium in health. As bacteria accumulate, all the way down the sulcular epithelium, which is a bioactive epithelium, it reacts by producing chemokines, which in turn stimulate inflammation within the connective tissues that we call gingivitis. In the so-called hyper-responsive periodontitis patient, that inflammation becomes uncontrolled and progresses, giving rise to attachment and bone loss. If you take oral epithelial cells in culture, such as this immortal cell line from Professor Stephen Primes lab in Bristol, and challenge the cells with, for example, killed *F. nucleatum* or *P. gingivalis*, you can demonstrate using a monoclonal antibody for NF-κB, translocation to the nucleus and pro-inflammatory genes being transcribed. The mRNA production for IL-1, IL-8, GM-CSF and TNF-α is shown here by reverse transcriptase-polymerase chain reaction. However, if you preincubate these cells with GSH, then what you find is a downregulation/switching off of inflammatory gene transcription.

So, I hope that gives you a taste of where we may be going hopefully in the future in terms of modulating the inflammatory response to bacteria and trying to dampen it down to reduce the exaggerated inflammation we associate with periodontitis.

As is always the case, I have to thank the people who have done all the work, which is of course not me, I just talk about it! I'd like to thank John Matthews, Paul Cooper, Helen Wright, Mike Milward, Anthony Roberts, Gareth Brock and of course Thomas Dietrich. I'd like to leave you with a taste of what the general public already know and understand about this subject. This was an article a couple of years ago in the Mail on Sunday, a paper in Britain, and it was basically asking or telling you how to fight stress and disease by monitoring your antioxidant levels, and what was really encouraging was that gingival bleeding was at the top of a list, far more important even than your sex drive, in the eyes of the Daily Mail at least!

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