Journal of Bioanalysis & Biomedicine

Loudon, J Bioanal Biomed 2013, 5:5
DOI: 10.4172/1948-593X.1000095

Research Article Open Access

Preventing and Correcting Communicable and Non-Communicable Chronic Disease via Amlexanox – Dual 'No-Nonsense' and Inflammatory Axis Targeting

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Abstract

In this sequel article on Amlexanox I investigate the multi-tasking potential for this drug, a recently discovered readthrough agent with immune-modulatory properties, for management of a wide range of human diseases including ageing modeled as a disease. The focus is not only on correction or disease rescue, but also on early prevention through use of Amlexanox prophylaxis. The concept of readthrough of nonsense mutations is further explored and correlation of nonsense mutation with cancer spread and stage is examined. Many other prevalent disease processes are examined in the light of nonsense-mediated causation, for example, intellectual disability and ageing. A primary aim of my current investigation is to show that both communicable diseases (related to infections from viral and bacterial agents) as well as non-communicable diseases (such as cancer, diabetes and inherited malformations/dysfunctions) may all form suited targets for Amlexanox therapy. As such, *ex vivo* and *in vitro* studies and animal models are discussed with the overall theme being to translate positive findings into the clinic. Clearly, this would have a major benefit with management in many inherited disease states and for infectious diseases. Further, a major benefit can be predicted for acquired chronic conditions too. The long understood property of Amlexanox in immune-modulation is exploited in this analysis. By acting through part-control of the NF-kappaB transcriptional factor-inflammatory axis, Amlexanox is capable of modulating the pathophysiology of such processes as cancer, vascular disease and diabetes with obesity. Moderating the response to pathogen challenge is a focus of attention in this present investigation. This is important insofar as Amlexanox mediates inflammatory-axis regulation and host-pathogen interactions, strongly suggesting that it must be explored in this context. As a result of this, interference with this arm of the innate immune system may well have consequences in terms of exposure to certain infectious agents. Detailed animal model systems as well as formal clinical trials are definitely called for to clarify the longer-term adverse reaction this may produce in the face of pathogen exposure. Amlexanox has been clinically approved for many years and, along with other drugs with similar immune-modulating capacity, appears satisfactory for long-term usage. Therefore, in practical terms, pathogen challenge in such a context may not pose significant threat. Overall, clinical trials are universally called for in order to ascertain the full potential for this old drug presenting with some exciting *'new tricks'*. I aim to be able to purposefully 'repurpose' Amlexanox and add this drug into the 'Doctor's bag' as a highly valuable medical adjunct to manage a wide plethora of medical conditions.

Keywords: Aminoglycoside, Amlexanox; Anthrax; Arthritis; Atheroma; Bacterial Infection; Bleeding Disorders; Cancer; Cancer Invasion; Cancer Metastasis; Cancer Stage; Cancer Therapy Resistance; Cardiac and Vascular Disease; Cerebral Palsy; Colon Cancer; Congenital Defect; Cystic Fibrosis; Cytokine; Cytomegalovirus; Dental Infection; Depression; Diabetes; Diabetes Insipidus; Duchenne Muscular Dystrophy; Epilepsy; Fungal Infection; Haematological Malignancy; Head And Neck Cancer; Herpetic Infection; HPV Infection; Ikkepsilon; Immune-Deficiency Virus; Infectious Mononucleosis; Inflammation; Influenza; Inherited Disease; Innate Immunity; Interleukin; Leukaemia; Lung Cancer; Mammary Gland Carcinoma; Measles; Mental Disease; Neurological Disease; NF-Kappab; Noncanonical NF-Kappab Pathway; Nonsense Mutation; Olfaction; Osteoarthritis; Osteodystrophy; Osteogenesis Imperfect; Osteoporosis; Osteosarcoma; Ovarian Carcinoma; Paget's Disease; Pain; Periodontal Disease; Proinflammatory Cytokine; Prostatic Carcinoma; Readthrough; Rickets; Scoliosis; Skin Cancer; Small Round Blue Cell Tumour; Taste; TBK1; Thalassaemia; Tnfalpha; Viral Infection

Introduction

In my previous article [1], I outlined an approach for nonsense mutation rescue with the new generation of readthrough agents, launched by Amlexanox. A broad repertoire of nonsense mutational gene targets was defined from cancer to heart disease to neurological disease. In this article, I aim to firstly further explore the usefulness of readthrough therapy by adding to the general repertoire of targets that would benefit from this powerful approach. I aim to go beyond non-communicable diseases and inherited conditions but venture into providing a focus too on communicable disease therapy. I summarize aspects of proof of principle that have been obtained with a variety of first (as represented by aminoglycoside) and second (as exemplified by Ataluren) generational readthrough agents in further support of this valuable readthrough protocol. I examine the significance of uncovered novel properties of Amlexanox other than readthrough. These aspects relate to modulation of the inflammatory axis and innate immune

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Received November 04, 2013; **Accepted** December 27, 2013; **Published** December 31, 2013

Citation: Loudon JA (2013) Preventing and Correcting Communicable and Non-Communicable Chronic Disease via Amlexanox – Dual 'No-Nonsense' and Inflammatory Axis Targeting. J Bioanal Biomed 5: 138-179. doi:10.4172/1948-593X.1000095

response. The relevance of modulating the innate immune system and inflammatory axis in respect to pathogen challenge is also explored.

Methodology

This investigation relied on a detailed and focused literature search of the PubMed medical database [2]. Search terms including phrases and individual keywords were linked with the Boolean operator *"and"* in order to select for articles containing all search terms. This protocol acted in a literature defining and limiting manner. Searches were aimed to be novel in the selection of terms employed in order to reveal insightful and new relationships from up-to-date published medical literature.

Articles were normally selected after the year 1990 and most frequently after the year 2000. The approach systematically scanned the literature for articles that corresponded to select subject topic headings. For exploring cancer stage and metastasis as relating to nonsense mutations the following search terms were employed: haematological malignancy/leukaemia and nonsense mutation and stage; colonic cancer/lung cancer/prostatic carcinoma/mammary gland carcinoma and nonsense mutation and stage/invasion/metastasis (representing the major solid malignancies). Other cancers examined included: mesothelioma/skin cancer/ovarian cancer/head and neck cancer/neurological, brain tumour/small round blue cell tumour/osteosarcoma and nonsense mutation and stage/invasion/metastasis.

Cancer therapy resistance and its potential relation to nonsense mutations was explored through the use of key phrases: cancer therapy resistance and nonsense mutation.

The analysis of nonsense mutation and neurological conditions was assessed by searches using key terms: mental disability/intelligence and nonsense mutation; depression/aggression/obsession-compulsion/mental illness/talent and nonsense mutation. In terms of bone disease, search terms used were:

Osteogenesis imperfecta/osteoporosis/rickets/osteodystrophy/Paget's disease/dentinogenesis/scoliosis and nonsense mutation. Joint diseases including osteoarthritis were examined via searching: arthritis and nonsense mutation. Disturbances in coagulation were studied through searching: bleeding disorders and nonsense mutation.

The involvement of a nonsense mutational event in disruption/alteration of either of the five senses was examined through searching: eye disease/hearing disorder/taste disorder/olfaction and nonsense mutation. For touch sense alteration the following was searched: pain and nonsense, as pain is frequently a reflection of the reaction of the skin to noxious stimuli.

Communicable/infectious-based diseases and their relation to nonsense mutations at both host and pathogen level were explored through searches employing the following key phrases: measles/influenza and nonsense mutation; herpes/cytomegalovirus/HPV/infectious mononucleosis/Immune-deficiency virus and nonsense mutation; bacterial infection/anthrax and nonsense mutation; fungal infection and nonsense mutation; dental infection and nonsense mutation.

Nonsense mutations as relating to diabetes were explored using key phrases: diabetes and nonsense mutation; diabetes insipidus and nonsense mutation; early onset diabetes and nonsense mutation. Further, searches examining 'conditions' such as ageing as a disease relating to nonsense mutations/inflammation utilized such example key phrases as: ageing and nonsense mutation; ageing and TBK1/ikkepsilon.

Exploring Amlexanox and its relation to the innate immune system and inflammation utilized these key terms: Amlexanox and inflammation and NF-kappaB (a central effector of the innate immune/inflammatory response). Amlexanox targets the NF-kappaB signaling axis via TBK1 and ikkepsilon noncanonical kinase inhibition. The relation between these functioning kinases and infections by bacteria and/or viruses and the importance of these kinases in directing host defense was explored. Searches used key terms such as: virus/bacterial infection and TBK1/ikkepsilon (along with alternative naming: IKBKE, ikki)/noncanonical NF-kappaB kinase. As an example of a chronic and prevalent bacterial infectious process, periodontal disease was selected. This was examined in terms of the significance host defense machinery/inflammatory response via noncanonical NF-kappaB signaling plays in this process. Key search terms used were: periodontal disease and TBK1; periodontal disease and ikkepsilon. As Amlexanox impacts on the NF-kappaB signaling axis then the importance of the relation between innate immunity (viz: led by the NF-kappaB) and vascular disease was explored through key terms: atheroma and NF-kappaB/TBK1/ikkepsilon/inflammation. Periodontal disease and heart disease are examined together by searching: periodontal disease and heart disease. The relation of the NF-kappaB axis in respect to both these conditions was performed using search criteria: periodontal disease and innate immunity/TLR (Toll receptor) and heart disease. Mechanisms of inflammatory cytokines as relating to coronary artery disease and periodontal disease were explored through examining individual key proinflammatory mediators. Examples are: TNF-alpha/interleukin-6 and coronary artery disease/periodontal disease. The important influence that proinflammatory mediators have directly on the heart was explored per the following example: cardiomyocyte cytoprotection and TNF-alpha/interleukin-6. This searching protocol was judged to be important as the specific interaction of inflammatory mediators needs to be examined in that particular context (viz: heart) directly to explain how inflammation impacts on the cardiac and vascular system. This in turn explains various clinical phenomena as relating to chronic inflammatory diseases. As Amlexanox impacts on the host inflammatory system it is important to explore the relevance of this influence.

Cancer too is explored in relation to the noncanonical NF-kappaB activation pathway via searches: cancer and TBK1/ikkepsilon/non canonical kinase to examine what impact modulation of this pathway by an agent such as Amlexanox may have in terms of cancer progression.

Novel combined literature searches using overlapping key terms: cancer and nonsense mutation and inflammation were also performed to delve into the literature as to extract information that may cast light on the effects of using a Readthrough agent which also possesses inflammatory/immune modulating capacity.

In terms of data analysis, abstracts of all cited articles were examined and, in addition, full length papers in a number of selected cases in order to examine the breadth of targets covered by Readthrough. Subject themes such as cancer and heart disease and rare and inherited diseases formed a particular focus. The concept of ageing as a disease was considered as a theme throughout and its relation to nonsense changes. The data extracted from the literature aimed to represent a fair and unbiased approach to examining the effects of long term nonsense rescue therapy combined with inflammation modulation.

Overall, the principle involved in this research was to scan in detail the medical literature, performing 'data recycling' – that is, examining prior literature by means of novel search criteria. Examining prior data by new means is commonly performed in science [3] and I have simply adapted that approach for my medical science research.

Results and General Discussion

Inspired by the work showing nonsense mutations associate with colonic cancer spread to the liver [4], as already outlined [1], I examined whether this was an isolated finding or makes a more generalised statement concerning a potential relationship/alignment between nonsense mutations and cancer stage/metastatic spread.

Stage of haematological malignancies and nonsense mutations

In an earlier article [5], I have discussed nonsense mutations for haematological malignancy (HM) and the rescue of these by Ataluren, effectively a second generational readthrough agent. Many blood cancers are aetiologically related to nonsense mutations (Premature termination codons - PTCs). In a study in myeloma cases where the majority of p53 tumour suppressor (TS) mutations were found to be PTCs [6], p53 mutation was correlated with poor survival and is associated with resistance to therapy. In myeloma, p53 mutations have been noted to widely vary in terms of prevalence [7]. Further, it has been stated that p53 inactivation by deletion or point mutation is an uncommon event in multiple myeloma and is more restricted to later stages of disease progression [7]. Mutations of p53 are seen at a level of 5% at diagnosis and 20-40% in advanced myeloma and at >60% in human myeloma cell lines (HMCLs). Cell lines may well perhaps reflect a more established form of cancer consistent with myeloma as a multistep process and p53 changes represent an important later-stage event in the established myeloma malignancy.

Inactivation of the p16 TS is also seen and is more prevalent with advanced stage of myeloma [7]. An epigenetic mechanism of methylation appears to be relevant in this context. Mutation of Retinoblastoma, pRb TS is a late event in myeloma, albeit rare. Inactivation of PTEN TS is seen in HMCLs and transfection of wild-type PTEN into HMCL inhibited tumour formation in mice [7].

p53 mutations, viz; point mutations and deletions, may be found in 13% of newly diagnosed cases of myeloma and form an independent poor prognostic factor - defining a particular 'high risk' cohort [6]. Of the nine presenting p53 mutations detected, three of these were PTCs in the DNA binding domain of p53 viz: p.Cys182x exon 5; p.Arg196x exon 5 and p.Arg213x exon 6. There were only 9/268 p53 mutated samples (3%) at baseline presentation. Nevertheless this study sets the stage for future studies comparing to more progressive disease with the percent and nature of p53 mutations involved in that progression. Nonetheless, patients with nonsense mutations had a shorter survival (median survival time for cases without p53 mutation is 41.4 months and with p53 mutation is 16.7 months). Thus targeted treatments early on as a means to halt progressive disease should certainly be initiated for nonsense codon mediated myeloma. It was pointed out that the drawbacks in studies have been that they tend to focus on limiting the analysis to exons 5-9 of p53 - thus potentially excluding valuable information as mutations that are relevant to cancer may occur beyond these exons [6]. Further, the point is made that mutational data gathered from newly diagnosed vs relapsed cases at initial presentation is important in order to take into account which mutated TS loci are relevant to advancing disease. Overall, in toto, it appears that the prevalence of p53 mutations (encompassing nonsense-type) increases with more advanced disease for this particular haematological malignancy. As the authors of the study say: 'the knowledge of the genetic abnormality that identifies this high-risk cohort may be exploited therapeutically by specifically targeting cells with TP53 abnormalities'. Indeed this is what I wish to achieve via readthrough therapeutics for nonsense mediated malignancies overall.

From COSMIC (Catalogue of Somatic Mutations in Cancer) one may appreciate the contribution substitution nonsense mutations in TS may make in regards lymphoid malignancies [1,5]. Mutation for one allele may be sufficient with a nonsense mutation to lead to haploinsufficiency, which, in turn, may be related to development of leukaemia. An example is the CDKN1B TS gene for T-cell prolymphocytic leukaemia [8]. In the absence of biallelic inactivation of CDKN1B there results preleukaemic clones arising in mutational animal models with one CDKN1B allele mutated. This is consistent with a haploinsufficiency mechanism in evolving this form of leukaemia. TS halploinsufficiency via nonsense mutational changes in one allele is linked to pathogenesis and although not a late stage event is still a necessary event nonetheless in the multiple genetic step pathway to cancer [8].

In development of adult T cell leukemia (ATL) an oncogenic role for human T cell lymphotropic virus type I (HTLV-I) Tax protein has been supported along with viral escape from host immune system [9]. PTCs in Tax gene are mutations that allow escape of HTLV-I from cytotocxic T lymphocyte (CTL) response in HTLV-I carriers - essentially functioning as 'escape' mutations. This 'escape' mechanism occurs at an early stage of ATL formation and host genetic changes (such as co-mutations in p53) combine to allow ATL progression [9]. Wild-type Tax binding to HLA A*02 elicits a strong CTL response. PTC in the 5' portion of Tax gene looses transactivation activity on its viral enhancer - an alteration found in four patients with ATL. So, in the development of ATL, HTLV-I infected cells can escape host immune system thus have an opportunity to gather further genetic changes and progress to malignancy, that is, acute ATL [10].

Further, HTLV-I induces cell proliferation and cell-to-cell transmission along with a long latency period. A fraction of carriers develop ATL and ~10% cases present with Tax gene mutations in ATL. Nonsense mutations in viral genes including Tax were seen in proviruses from carriers suggesting these mutations are generated through reverse transcription and prior to oncogenesis (ATL). Mutational induction during transcription is a survival mechanism used by the virus HTLV-I to escape host immune response by functional loss of viral proteins [11]. Restoring function by readthrough could well be seen to be beneficial in preventing disease progression.

Congenital neutropenias may degenerate into myelodysplastic syndrome and acute myeloid leukaemia (AML). Disease progression has been, in part, associated with mutations in GCSF-R (Granulocyte Colony Stimulating Factor Receptor). Notably, in a subgroup of patients, nonsense mutations in GCSF-R were found and these cause a truncation of C-terminus – a domain needed for G-CSF induced cellular maturation – these patients are predisposed to develop AML [12]. In congenital inherited neutropenia there is a maturation arrest at promyelocyte and myelocyte stage in the marrow and absence of mature neutrophils. GCSF-R mutations are highly clinically relevant in that they induce maturation arrest. It was noted that four out of twenty eight patients with congenital neutropenia possessed a point mutation

within the cytoplasmic region of GCSF-R - typically replacing a glutamine residue with a stop codon. Importantly, two of these cases developed AML. Recombinant G-CSF therapy was given and mature neutrophils developed in response. Notably too, GCSF-R mutations examined were in fact sporadic rather than inherited and this adds to the spectrum of this disease [13]. It was not therefore considered that the Receptor mutations were causative of the neutropenia as response to recombinant GCSF was seen. Nevertheless, it still remains to be shown that rescue of the Receptor mutation may prevent a leukaemic clinical course – Readthrough could well serve a purpose in this respect. A clear mechanism of how the Receptor mutations may lead to leukaemic transformation needs to be elucidated as response to GSCF is seen. More recently, it is re-iterated that truncating mutations within the granulocyte colony-stimulating factor receptor are associated with the formation of myelodysplasia/AML within the context of congenital neutropenia [14]. As stated, a mechanistically-based causal relationship between Receptor mutations and leukaemic degeneration has not been clarified. Truncated Receptor interacts with the PML-RARalpha oncogene to produce AML in mouse model [14]. Production of the truncated Receptor greatly shortens the latency of AML and is associated with a particular AML with high blast counts and severe myelosuppression. These data certainly tend to support a causal relationship between Receptor mutations and AML. It is therefore logical to assume that nonsense-mediated Receptor truncating mutations found in clinical samples may be acting in a similar fashion to this experimental system. Indeed if that is so, then readthrough has a significant place within the field of AML prevention in the backdrop of congenital neutropenias.

In five out of ten leukaemic human T - cell lines examined by cDNA PCR-amplification both nonsense and missense-type p53 mutations were revealed [15]. It ought to be emphasized that cultured leukaemic T cells represent a late stage in the leukaemogenic pathway as has been stated above [6]. This is oftentimes the case particularly where cell lines are established from patients in leukaemic relapse phase. Mutation of one or both alleles of p53 may reflect selection of cells with increasingly tumorigenic phenotype [15]. The cell line HUT78 possesses a homozygous p53 stop mutation at codon 196 (p.Arg196x). This mutation most likely has arisen *in vivo* during the process of relapse - a late stage in leukaemia - perhaps following the loss of wild-type allele followed by a duplication of the chromosome with the mutated allele. HUT78 also lacks TS pRb suggesting that both pRb and p53 TS serve as gate point TS in determining the fully tumorigenic leukaemic T cell state. The authors state that it may be relevant and important to reintroduce wild-type p53 into cell lines which have p53 mutations to fully assess p53 role. In respect to the cell line, HUT78, Readthrough of the homozygous nonsense mutation in p53 would serve as an excellent *ex vivo* model to examine the importance not only of p53 as a control point or gate in the late phase leukaemic process but also to test the efficacy of selected Readthrough drugs. In this context, my suggestion would be to examine Amlexanox against this target - a theme I pursue throughout this article.

A novel nonsense mutation was found at p53 codon 213 in a case with CML (chronic myeloid leukaemia) in blast cell crisis [16]. Patients with nonsense and indeed other mutations which scored as missense were in an advanced disease state. Although infrequent, inactivation of p53 is certainly apparently significant in the disease process/progress of several types of haematologic malignancies [5].

In two out of one hundred twenty eight (2/128) patients with AML/ALL (acute lymphocytic leukaemia) and MDS-myelodysplastic syndromes/CLL (chronic lymphocytic leukaemia) DNA analysis showed a p53 mutation with negative immunohistochemistry. This is consistent with a nullifying nonsense mutation with reduced expression of truncated p53. It ought to be noted that wild-type p53 has a short half-life and also scores negatively on immunohistochemistry. Misssense-mutated p53 has a prolonged half-life and becomes detectable on immunohistochemistry. Thus, special immunological stains on blood and bone marrow smears is a sensitive method for p53 mutation detection in haematologic malignancies, though firstly one must rule out PTCs to avoid confusion with the wild-type protein [17].

RUNX1 is needed for normal haematopoiesis and I have already summarized that RUNX1 is a TS [5]. RUNX1 mutations are present in CMML – chronic myelomonocyctic leukemia. Thirty two RUNX1 mutations were found in 30 of 81 patients presenting with CMML. These alterations were located in the N- or C-terminal portion of RUNX1. Seven nonsense mutations were isolated and there was a trend for higher risk for AML progression in mutation positive cases – especially where the C terminus is affected/truncated – which logically includes all nonsense mutations. Median time to AML progression was 6.8 months for C-terminal mutations and 28.3 months for those without C- terminal mutations. Truncating mutations can therefore be assumed to be associated with more frequent and rapid AML progression [18].

Stage of solid malignancies and nonsense mutations

The four most commonly presenting cancers: viz: lung, colon, mammary and prostatic carcinoma are considered in terms of their stage of presentation and relation of this to TS substitution nonsense alterations. Other cancers are also considered in respect to this relationship to provide an overall impression of the tie between this type of mutation that forms a suited Readthrough target and stage, which in turn reflects prognosis and treatment outcomes.

Colonic cancer: Cellular SRC – cSRC – human homolog of Rous sarcoma viral oncogene (v-src), is activated in colonic cancer [19]. A subset of colonic cancers possess a nonsense mutation at codon 531 which truncates cSRC directly C-terminal to the cSRC kinase regulatory domain. This mutation is activating and transforming and promotes metastasis. Such a paradoxical activating truncating mutation is reminiscent of activating NOTCH mutations via truncation of the PEST domain that leads to activation [1]. SRC is a non-receptor tyrosine kinase. Residue Tyr530 in human SRC is a site for phosphorylation that acts to downregulate activity. A truncating mutation at codon 531 of SRC in ~12% of cases of advanced human colonic cancer is consistent with the proposition that this mutation activates SRC, which, in turn, promotes metastasis [20]. This makes several key points, viz: it is not just inactivating truncating mutations in TS that pave the way to cancer progression, activating mutations in oncoproteins are also involved. Further, readthrough could prove to be a most useful approach and strategy to prevent colonic carcinoma metastasis in the backdrop of nonsense mutations either inactivating key TS or activating oncoproteins.

Sporadic somatic mutations of p53 occur in adenomatous polyposis cases. In ampullary carcinoma, (a malignant cancer from the Ampulla of Vater, the last section of the common bile duct), five of twelve p53 mutations analyzed in this tumour were nonsense mutations – a relatively high percentage. APC gene alterations were also seen, although not nonsense codon alterations. Thus changes in the APC

and p53 genes are quite common in sporadic ampullary cancers [21] – with readthrough having a potentially very significant role to play in rescuing p53 nonsense changes found in this uncommon, but serious gastrointestinal cancer.

Ulcerative colitis is a form of inflammatory bowel disease in the same class as Crohn's disease, a presentation typical of immune dysfunction. Treatment is oftentimes with anti-inflammatory agents. Such inflammatory diseases with immune dysregulation prove to be risk factors for colonic cancer development. p53 point mutations may be seen in the context of ulcerative colitis-associated colonic cancer [22]. In a significant proportion of cases, nonsense mutations are evident suggestive of being mechanistically involved in malignant progression of ulcerative colitis. As oppose to sporadic colonic cancer where nonsense p53 mutations are related to metastatic liver spread [1,4], ulcerative colitis-associated neoplastic progression may involve p53 inactivation at relatively early, noninvasive stages – thus setting the scene as it were for malignancy in this situation. Clearly, rescuing nonsense mutations early on in the disease stage or prophylactically in the context of ulcerative colitis could have very promising clinical appeal. Again, Readthrough with an accepted agent such as Amlexanox has a great potential in this regard, a theme I pursue throughout this presentation.

As already outlined [1,4] substitution nonsense mutations appear rather prevalent in p53 TS in colonic metastases to the liver as oppose to primary cancers. This observation rates readthrough as a very appropriate technique in prevention of colonic cancer spread – which typically occurs to the liver. The liver is significant in oncology as this organ commonly forms not only a source of primary cancerous growth as represented by hepatocellular carcinoma but also a site for metastasis – particularly for gastrointestinal tumours. Indeed, as already mentioned, there is a high percentage incidence of PTCs in liver metastases of colonic carcinoma [4]. The percentage of nonsense mutations are >3-fold higher in liver metastasis over primary colonic cancers. It is considered on the basis of this observation that loss of p53 is a causal step in malignant progression rather than in the initiation of the cancer process itself. In most cases (~90%) with PTC in p53 there was also loss of the second allele - as demonstrated via loss of heterozygosity (LOH) at the p53 locus at 17p. As such, truncating mutations could produce a totally null phenotype for p53. Mechanistically, it was explained that there is a functional variation between missense and PTC mutations in p53. Missense mutations within exons 5-8 perturb the DNA binding action of p53, leaving other domains intact. However, PTCs result in loss of the C-terminal region which includes other domains such as the tetramerization domain, apoptotic domain and nuclear localization signal region. Therefore, PTCs create a protein that is more seriously 'deformed' as it were, compared to a mere missense distortion of the DNA-binding domain.

Supporting these observations are studies with the SMAD4 factor. SMAD4 is a 552-amino acid protein involved in cell signaling and modulates members of the TGFbeta superfamily of proteins. SMAD factors are conserved across species and particularly so within the N-terminal MH1 domain and C-terminal MH2 domains. MH1 domain has DNA binding properties and MH2 is responsible for cognate receptor binding as well as DNA binding and oligomerization with other SMADs. As SMAD4 is oftentimes found mutated in several cancers it is considered to be a TS via TGFbeta regulation. I have previously noted that SMAD4 is a TS that, when mutated by germline inactivating nonsense alterations, may result in gastric juvenile polyposis – a precancerous condition [23]. Many invasive and metastatic colonic carcinomas carry SMAD4 mutations [24]. SMAD4 truncating mutations - substitution nonsense– in one allele (with loss of the other allele), are seen in a significant proportion of cases with invasive colonic cancer and metastasis which certainly suggests a causative link. Overall, SMAD4 inactivation is concluded to be involved in advanced stages of this disease [24]. Loss of heterozygosity is seen in colonic cancer at 18q [25]. In two cases with LOH, substitution nonsense mutations, viz: p.Arg445x in exon 10 and p.Glu538x in exon 11 were found [25]. These cases represented advanced disease. In toto, these observations suggest that, as above [24], SMAD4 inactivating mutations are associated with advancing cancer of the colon and that readthrough therapy would logically be able to target the proportion of cases arising from nonsense codon changes to prevent advanced colonic carcinoma.

Lung cancer: p16 TS status was examined In thirty primary non small cell lung cancers (NSCLC) with metastatic spread to thoracic lymph nodes and thirty three NSCLC minus nodal spread [26]. Four nonsense and one frameshifting mutation were found in addition to a deletion. Mutations were confined to the group of cancers with lymph node metastasis, presenting in both primary and nodal spread. No p16 mutation was found in tumours without nodal spread. Grade, primary size and age of patient did not correlate with mutational distribution – only metastatic spread. p53 TS mutations were found in a proportion of cases demonstrating nodal spread as co-mutants. The significant percentage of nonsense mutations in p16 certainly suggests that readthrough could have benefit for prevention of metastatic NSCLC within this patient cohort.

CDKN2 TS, an inhibitor of type D cyclin dependent kinases, was examined via PCR-SSCP analysis in NSCLC samples (71 cell lines and 54 tumours). Six of the cell lines and four tumour samples demonstrated point mutations - all were from advanced stage III or IV disease. Two cases with nil TS mutation in primary cancer had TS mutation in metastatic NSCLC cancer. Mutations were coding strand G:C to T:A transversions in 5/10 point mutations – 7/10 were nonsense codon changes [27]. The significant correlation of advanced disease with TS CDKN2 nonsense mutation is plain to see and forms an excellent target for readthrough strategies aimed to reduce this cancer's advancement.

Prostatic carcinoma: The DU145 prostatic carcinoma cell-line derives from a brain metastasis. This particular cell line was examined with techniques such as nonsense-mediated mRNA decay microarrays and array-based comparative genomic hybridization, as recently reviewed by myself [1]. By this method, genome-wide identification of genes with allelic inactivation with nonsense mutations and loss of wild-type allele may be readily sought. As such, an allelic truncating mutation of EphB2 - receptor tyrosine kinase - with deletion of second allele was identified in DU145. Transfection of this cell line with EphB2 protein expression constructs decreases cell line survival. This represents a 'proof of concept' in that EphB2 inactivation is associated with progression and metastasis of prostatic carcinoma [28]. Since nonsense codon inactivation of EphB2 is likely to be related causatively to the metastatic potential of these carcinoma cells then readthrough again has a clear significant potential in the armamentarium against the cancerous spread of this disease.

A nonsense codon founder mutation in BRCA2 gene (p.Glu1953x) appears with early-onset aggressive-type prostatic carcinoma in a French-Canadian family [29]. BRCA2 p.Glu1953x appeared in a proband with extensive affected family history of five brothers, father, and uncles on the proband's father's side. Notably, treatment included:

radical surgery/hormone-therapy/palliative-chemotherapy/genetic counseling. To this I would advise adding readthrough – certainly an important consideration for medical doctors to add to their armaments against this and other cancers [1]. Interestingly too, the features and presentation spectrum of prostatic carcinoma in this family is consistent with a diagnosis of familial prostatic carcinoma – not sporadic. In fact, earlier it has been noted that familial cases of prostatic carcinoma may be considered a disease in its own right [30]. This cancerous process demonstrates a higher treatment failure rate due to heightened distant spread relapse rates and overall more aggressive disease.

Mammary carcinoma and nonsense mutation and stage: In one hundred and eighty cases of mammary carcinoma, 32% showed p53 mutations and half were nonsense/insertion/deletion/splice site alterations. Mutations in p53 correlated with poor survival overall in node (+) cases but nil correlation with survival was seen in node (–) cases. Thus p53 mutational status, size of primary and nodal involvement was significant attendant and correlating features [31]. One may conclude that nonsense alterations in p53 have a connection to poor survival via potentially relating to nodal spread with other factors being involved. In this regard, readthrough may have a place too in mammary carcinoma management.

In a case with nonsense BRCA1 gene mutation in exon 13 the presentation was that of an aggressive lesion of early onset with ductal infiltrating carcinoma with positive axillary node [32]. This mutation has a distinct inherited background with other close relatives affected (mother, sister and niece). In another instance, the BRCA1 germline nonsense mutation p.Q563x may be found in cell line: L56Br-C1 established from axillary node (+) tissue. Nil BRCA1 protein is found in this cell line. Other mutations include a missense p53 alteration along with decreased expression of epidermal growth factor receptor and keratin 8. cDNA microarray profiling of global gene expression comparing primary tumour and lymph node metastasis and cell line demonstrates a correlating pattern of gene expression [33]. Therefore this cell line represents fairly accurately, it would seem, the *in vivo* cancer cell phenotype and as such, may be very useful as a model for testing and examining new therapies for nonsense mediated BRCA1 induced mammary gland carcinoma – such as Amlexanox readthrough. I have already noted the relationship of nonsense mutations in BRCA along with the proposal for rescue of such pathogenic mutations via Readthrough [23]. This readthrough therapy concept now would appear very promising and relevant in terms of actual direct clinical application in mammary gland carcinoma.

Nonsense mutation and mesothelioma and stage: BRCA1 associated protein 1 – BAP1, is a deubiquitinating enzyme, and regulates histone ubiquitination. Ubiquitination is a post-translational modification whereby ubiquitin is added to the protein substrate. This may in turn lead to signaling for the protein's degradation via the proteasome or alter protein interaction. BAP1 is a TS and metastasis suppressor. In a case of mesothelioma, a nonsense mutation has been identified in BAP1 [34]. Germline BAP1 mutations may predispose to mesothelioma as well as other cancers, such as melanoma [35]. A family with a germline BAP1 nonsense mutation (p.Y241x) demonstrates a proband with uveal melanoma. The proband's father has mesothelioma and cutaneous melanoma and a paternal uncle had lung cancer, cutaneous melanoma and uveal melanoma. Thus this is best described as a BAP1 Cancer syndrome – predisposing to lung cancers and other cancers beyond the lungs.

The NF2 gene is somatically mutated in mesothelioma [36]. Two mesothelioma cell lines had nonsense mutations at codon positions 57 and 341 of NF2. Mesothelioma cell line: NCI-H2052, possesses the p.Arg341x mutation which is a somatic change. The cell line, HP-3, possesses p.Arg57x stop mutation. NCI-H2052 is from metastatic site lymph node - stage 4 mesothelioma. Thus NF2 is important in the oncogensis of a subset of mesotheliomas but contrasting to this, mutations were not found in any lung cancers such as with carcinoids, NSCLCs and small cell lung cancers. These cell lines, viz: HP-3 and NCI-H2052 may certainly provide fruitful tools for *ex vivo* experimentation to aim in demonstrating proof of principle of readthrough for management of mesothelioma. Further to that, clinical trials with nonsense-mediated mesothelioma would be indicated with readthrough drugs, such as Amlexanox.

Nonsense mutation and skin cancer and stage: As presented above, germline BAP1 mutations may predispose to various cancers, including melanoma [35]. BAP1 is a TS and metastasis suppressor with exome sequencing providing evidence for inactivating mutations in BAP1 in uveal melanomas. Mutations in BAP1 are highly correlated with metastasis [37]. Disrupting somatic mutations in BAP1 occur in 26/31 metastatic uveal melanoma and 15 mutations result in PTCs. So BAP1 is a clear candidate TS which is frequently inactivated in metastatic melanoma. Further, a high percentage of disrupting mutations in BAP1 are truncating and these truncating mutations were associated with very much lower mRNA levels. This suggests nonsense-mediated mRNA decay (NMD) is important and involved in decreasing BAP1 protein levels. Nonsense substitution mutations even if not affecting a catalytic domain may cause decrease in mRNA level and overall protein inadequacy. These mutations certainly form an excellent target for readthrough therapies – particularly with a method that may also inhibit NMD – such as with Amlexanox treatment [1].

MITF – microphthalmia-associated transcription factor – is a basic helix-loop-helix leucine-zipper protein and a regulator for growth, differentiation and prosurvival of cells of melanocytic lineage [38]. As such, this factor regulates multiple downstream targets. Melanoma progression, albeit complex, involves notable changes in key regulators of expression of genes needed for melanocyte development and regeneration such as MITF. In fact, this transcriptional factor has been found amplified in a significant number of melanoma metastases and MIFT controls the cell cycle and its removal alters cell cycle control. Proliferative but not metastatic melanomas have activated MITF via Wnt pathway. Melanomas progressing to metastatic yet not in full proliferative mode had inhibition of Wnt pathway and down regulation of MITF. These findings are consistent with Wnt being a prosurvival factor for cancer as I have already discussed [39]. The transcription factor, Sox10, acts upstream of MIFT and synergizes with MIFT and >9% of primary melanomas have MIFT and Sox10 mutations and ~22% of metastatic melanomas carry these changes. In metastatic melanoma, Sox10 presented with three truncating mutations one of which was a substitution nonsense mutation - MIFT had nil truncating-type mutations. These findings may certainly suggest that joint interacting MIFT/Sox10 mutations correlate with advanced melanoma disease and that truncating mutations could play a role in the more progressive cases [38]. Again, supporting a role for readthrough – a therapy which may well be indicated in future therapies for melanoma.

Whole genome sequencing of matched primary and metastatic acral melanomas show that there is a close match in their mutational spectrum [40]. Few mutations are independent from being both present in primary and metastatic lesions. The authors state that, like

other cancers such as mammary gland carcinoma and pancreatic cancer, most of the original mutations in the primary are propagated into the metastasis. Notably, nonsense mutations were shared between primary and metastatic tumours in: DROSHA (p.Q1087x); ERCC5 (p.E418x); LRRK1 (p.E880x); LRRFIP1 (p.R380x). DROSHA encodes for a nuclease in microRNA processing and is linked to cancer - reduced DROSHA expression being associated with poor prognosis in neuroblastoma and ovarian carcinoma. ERCC5 is a component of nucleotide excision repair pathway and germline mutations are associated with xeroderma pigmentosum, [23], which has a 1000-fold increased risk for melanoma. Deficient nucleotide excision with decreased cyclobutane pyrimidine-dimer repair due to ERCC5 mutation may predispose to UV affects in the acral location. Further, ERCC5 mutations are associated with microsatellite instability in colonic and gastric carcinomas. LRRK1 regulates EGFR-trafficking and is associated with human cancers. Germline mutations are linked to Parkinson's disease and confer an increased risk for melanoma. LRRFIP1 acts to repress TNFalpha expression as well as EGFR. Finally as the authors say: "The heterogeneity inherent in these tumors (melanomas) could present a challenge in the era of targeted therapy'. Undoubtedly this is true, but as readthrough is not gene-specific, rather mutation-specific, then mutations in DROSHA, ERCC5, LRRFIP1 and LRRK1 that are substitution nonsense and noted in this study form excellent targets for readthrough and may produce significant benefits to reduce this disease process.

Loss of p53 is a step in malignant progression of chemically-induced skin cancer rather than initiation/promotion [41]. As such, one may presume that PTC inactivating mutations in p53 would represent an important step in progression/advancement of stage of this cancer, rather than forming an initiation mechanism in itself. Readthrough again may then be of use to prevent advancing lesions which would be of central concern in management.

Nonsense mutation and metastasis and ovarian cancer: Ovarian carcinoma generally arises from the surface epithelium of the ovary. Most cancers are detected late due to the subtle nature of the symptoms. A family history increases the risk by >9 fold and has been discussed in regards to BRCA1 mutations which are involved in mammary gland carcinoma-ovarian carcinoma inherited cancer syndrome complex [23]. Ovarian cancer is one of the most common gynaecologic cancers and one that carries a poor prognosis. In serous ovarian carcinomas, nonsense mutations in p53 have been noted to be not uncommonly present [23]. Therefore, p53 dysfunction appears rather prevalent in ovarian cancer. The complete coding sequences of one hundred and thirty tumour DNAs were examined via single-strand conformational polymorphism analysis and 32/94 cancers carried p53 null mutations, viz: six nonsense, twenty three frame shift and three splice-site changes [42]. Twenty eight patients in the study group had metastatic disease and distant spread was ~8-fold more likely present in patients carrying a null p53 mutant as compared to missense alterations or wild-type. Although most of the terminating mutations were frame shifts due to insertions/deletions, one substitution nonsense mutation, viz: p.Gln165x, was seen in a case with metastatic disease to the brain. Mechanistically, null mutations may be resulting in severe disruption of the TS protein structure and also producing NMD rather than missense mutations which only result in minor deformation in the protein's activity. Alternatively, the correlation between p53 null mutations and aggressive tumour behaviour, as demonstrated by distant metastases, could be downstream of more basic/fundamental molecular alterations. Genomic instability corresponds to development of p53 frameshift mutations and so genomic instability could well be the cause of the p53 mutational event rather than as a result. Genomic instability correlates with distant spread in various other cancers as well. Another concept is that of tumour vascularity. Protein p53, in part, regulates vascular proliferation via VEGF and thrombospondin. Complete failure of p53 null mutations to suppress VEGF with thrombospondin loss could well explain the ability of these tumours to spread. No patient with wild-type p53 or missense mutation had a distant metastasis. Overall, there was a significant correlation with null mutations and lymph node metastasis, advanced disease with high grade, and distant spread occurring early on in the disease presentation. Clearly, this has very important implications in regards therapy selection for this cancer. Readthrough could well prove to be a major step forward for management of this serious gynaecologic cancer in the years to come. In fact, the authors conclude by saying that: 'When null mutations are encountered, consideration should be given to novel treatment approaches including systemic p53 gene replacement therapy' – it may well be that readthrough with NMD inhibition would be able to achieve such an aim in a simple and safe fashion with a drug such as Amlexanox.

Nonsense mutation and Head and Neck cancer and stage: In a patient diagnosed with oesophageal cancer a nonsense mutation was found in p53 - exon 10. Genomic DNA was examined from 150 excised lymph nodes and PCR identified the same p53 mutation in the nodes. One + node was located along the right recurrent laryngeal nerve where nil positive nodes were found via routine histopathology. This case illustrates the use of PCR for p53 to aid diagnosis of minimal residual metastatic disease in addition to supporting the contention that p53 null mutations are a hallmark of oesophageal carcinoma [43]. It is possible that this mutation type may predispose to advancing disease although further studies need to examine genetic risk factors in detail for advanced oesophageal carcinoma.

Protein p53 nonsense mutations have been earlier found in Head and Neck cancer and are present in both primary and metastatic disease [44]. This is consistent with a model proposing clonal mutational dissemination from primary to metastasis. Authors suggest too that tobacco carcinogen is considered likely to have resulted in the transition and transversion mutations observed - as this is in itself a key risk factor in Head and Neck carcinogenesis. One conclusion of note from this analysis is that as the spectrum of p53 mutations was similar from primary cancers to metastatic foci then these alterations do not appear to be specific to metastasis formation. This however does not rule out the possibility that p53 null mutations occurring early on within the primary focus may be sealing the fate or destiny of such carcinoma cells to spread. Further examination of the role of specific TS mutations are required in Head and Neck cancer as relating to cancer spread and aggressiveness. Nevertheless, readthrough can be envisaged as potentially having a clear role to play in managing Head and Neck cancer.

To examine the role of the SMAD4 TS in Head and Neck carcinoma sixteen cell lines from eleven patients and twenty primary Head and Neck carcinomas were scrutinized. All coding regions were sequenced for SMAD4 in each case [45]. A nonsense mutation, p.Gln526x, was located for SMAD4 in two cell lines, viz: UMSCC22A and UMSCC22B. These were from primary tumour and lymph node metastasis of the same patient. It can be concluded that SMAD4 is involved in tumourigenesis in a subset of Head and Neck cancers. Further, nonsense mutations in SMAD4 may well destine cancer cells

to spread – this contention requires further experimental evidence - perhaps through using a cell line with nonsense mutation in SMAD4 and rescuing its activity via readthrough and testing *in vivo* with animal models to examine its relation to metastatic potential. Again, similar to the situation with p53 and Head and Neck carcinoma readthrough in this context could well have a significant place in future therapies.

Nonsense mutations for a key TS, viz: p53, may also be seen in precancerous lesions of the mouth limited to the epithelium in addition to being present in frank squamous cell carcinoma [46]. This suggests that at least in a certain subset of mouth cancers that nonsense mutations in TS may present early on in the mutli-step evolutionary process of carcinomatous change. This would allow clinicians a useful 'window of opportunity' to trial readthrough as a preventive strategy prior to development of invasive malignancy. Understandably then, the mouth offers an excellent environment for prophylactic and topical therapeutics.

Although smoking is certainly suggested as a prime risk factor for Head and Neck cancer – it may also predispose to other cancers such as those of the bladder [47]. Notably, TS p53 point mutations occurred in smokers - not in nonsmokers with early bladder cancer. Extended time frame for smoking was required over a number of decades to produce such changes. The p53 alterations were in early-stage/superficial bladder carcinoma cases mimicking somewhat the situation seen with mouth precancerous lesions (above). Again, readthrough therapy for nonsense mutation-mediated bladder cancer may have an impact in this tumour also – particularly if therapy is commenced early.

Nonsense mutation and neurolgical tumour and stage: I have presented some discussion previously regarding TS truncating mutations and neurological tumours [1]. A patient with two independent appearing meningiomas demonstrated nonsense mutational alteration in exon 8 of NF2 [48]. This alteration was the same genetic change in both tumours and is not a germline mutation. Given that the family history is negative for neurofibromatosis type 2 then it is reasonable to conclude that this represents a sporadic mutational event, consistent with a monoclonal origin of the presenting meningiomas. Early subarachnoid spread could be considered a mechanism for enabling the noted disease presentation. It is possible then to link NF2 nonsense mutational events to spread of this tumour.

Truncating mutations in NF2 correlate with an overall increased severity of presentation of tumours in patient cases [49]. Missense mutations appear as a relatively milder form of the disease process. Again, this emphasizes the theme of genotype-phenotype correlation and how readthrough may yet again prove to be clinically valuable.

Severe neurological disease is a presentation one may encounter associated with fulminant courses of Li-Fraumeni syndrome. In one example, a de novo germline p53 mutation was isolated in a patient with various synchronous brain tumours including primitive neuroectodermal tumour and choroid plexus carcinoma [50]. A nonsense mutation, p.R342x, was defined in the oligomerization/nuclear export signal domain. Readthrough could be considered for such cases to decrease their risk of deterioration due to neurological involvement by cancer.

Small round blue cell tumours and nonsense mutation and stage: Small round blue cell tumours (SRBCT) form a group of malignancies that appear as a group histologically similar - that is, as small, rounded cells staining blue by routine histological protocol since they possess merely scant cytoplasm. Generally they are paediatric population based. Example tumours in this group are Ewing's sarcoma, neuroblastoma, medulloblastoma, rhabdomyosarcoma, small cell lung cancer, Wilm's tumour and small cell lymphoma. Examples from this group where nonsense mutations play a potentially significant causative role are given here – it ought to be noted that this list is far from exhaustive however. In rhabdomyosarcoma, a significantly common soft-tissue paediatric sarcoma, a nonsense mutation was found at codon 80 of p16 TS [51] in an alveolar rhabdomyosarcoma cell line. Having said this, reduced expression of p16 appeared to show nil correlation with clinic-pathologic parameters. Other alterations were proposed to be acting in this instance. A case of a paediatric patient presenting with germline p53 nonsense mutation (p.R342x) with both primitive neuroectodermal tumour along with choroid plexus carcinoma has been outlined [50]. Metastatic alveolar rhabdomyosarcoma developed in this case. Investigation of this Li-Fraumeni-type case indicated that synchronous SRBCTs may occur, viz: primitive neuroectodermal tumour and rhabdomyosarcoma – as relating to nonsense mutational events. Further, fulminant course or advanced stage disease may correspond to such truncating p53 TS disruption.

Wilm's tumour is a cancer of the kidneys in the paediatric population generally. Bilateral (extensive) Wilm's tumours are found to be related to early presentation clinically and possess a high frequency of WT1 gene nonsense mutations in exon 8 [52]. This is suggestive that nonsense changes relate to advanced disease for SRBCTs.

Ewing's sarcoma is a rare condition whereby malignant cells are found in bone or in soft tissues. A nonsense mutation in TS p16 was found in Ewing's amongst other mutations and disease severity correlated to p16 mutational alteration [53]. This suggests that disruptive, truncating mutations in TS have a significant impact on disease pathogenesis for Ewing's SRBCT.

In neuroblastoma, another SRBCT considered to arise from autonomic cellular precursors within the developing neural crest, causative defining mutations in PHOX2B have been found [54]. The PHOX2B protein is significantly anti-proliferative in its action. Nonsense mutations, by disruption of PHOX2B function may be therefore considered predisposing to neuroblastoma formation [54].

Overall, nonsense mutations play a rather important role in causation of SRBCTs.

Osteosarcoma and nonsense mutation: Osteosarcoma patients with nonsense mutations in TS p53 tended to be younger at presentation than those with missense alterations [55]. Having said this, p53 mutations are nonetheless not late developing events in osteosarcoma progression – rather, they are seen prior to metastasis formation [55].

Rothmund-Thomson syndrome - RTS – is a genodermatosis - an inherited skin condition – with facial rash and short stature and sparse head hair/eyelashes/eyebrows. Skeletal changes with premature ageing and cancer predisposition, viz: osteosarcoma are seen. The condition is autosomal recessive and genetically heterogeneous. One gene involved is RECQL4 - helicase (ATP-dependent DNA helicase Q4). Frameshifting and nonsense and splice site mutations oftentimes prevail for RECQL4 alteration in this disease context [56].

Marfan syndrome, a disease affecting 1/5000 people is due to mutations in fibrillin 1 gene. A case with Marfan and osteosarcoma presented with a 2 bp insertion in Fibrillin causing a PTC [57]. Osteosarcoma cells derived from the case formed a cell line which assembles fibrillin 1 microfibrils in culture. Authors state that this cell

line (PSU-OS-M) is beneficial in that it may be used for nonsense-mediated Marfan Syndrome study.

As I have indeicated [23], a p53 stop codon in exon 5 was found as a somatic alteration in a paediatric patient with osteosarcoma of the tibia. The p53 mutant lacked transcriptional activity for p21 and bax target genes [58]. Further, as I have stated [23], a case with choroid plexus papilloma presented earlier with osteosarcoma. Analysis showed a 7 bp insertion in exon 5 of p53 generating a PTC at codon 182. Mutant protein was expressed at very low levels and failed to transactivate p21 or bax [59]. Surprisingly, this truncated TS was still capable of producing apoptosis – although other properties appear altered significantly. Again, readthrough may have a place in tackling selected nonsense-mediated osteosarcoma cases.

Overall, my literature investigation in this section has demonstrated that nonsense mutations oftentimes align with increased stage/malignancy for a variety of haematological as well as solid-based cancers. This analysis and presentation serves as a useful and novel analysis of PTC events in oncology and is certainly relevant in the context of readthrough technology (Figure 1).

Cancer therapy resistance and nonsense mutation

One may pose the question – do nonsense mutations producing in frame stop codons relate to development/alteration of cancer therapy resistance? Ovarian cancer cell lines taken at initial diagnosis vs post-chemotherapy relapse showed termination codons in p53 to be present in either type of isolated cell line. Cell lines bearing p53 nonsense mutations such as TOV3133G, TOV3133D (both from primary cancers), OV3133R and OV3133R2 (both from chemotherapy-relapsed cases) are nonetheless good resources for applying readthrough to examine *in vitro* response of these serous ovarian carcinoma cell lines [60] and examine chemotherapy effects in conjunction with readthrough.

Whole-genome sequencing was used to explore the mechanism behind significant and durable remission for metastatic bladder cancer in a case treated with Everolimus, an mTOR signaling pathway inhibitor. A mutation was found in TSC1 (tuberous sclerosis complex 1) - a loss of function. TSC1 mutation - correlates with Everolimus sensitivity [61]. In this scenario, readthrough may paradoxically enhance resistance to this drug and so again, this emphasizes the highly individualized nature of readthrough for cancer. Clearly, one needs to take into account drug sensitivity as an important aspect. On the other hand, nonsense mutations in CHEK2, which codes for CHk2, correlate with resistance to anthracycline therapy in cancers containing a wild-type expression of p53 [62]. CHk2 produces a protein kinase activated in response to DNA damage and is involved in cell-cycle arrest. It is thus a potential TS candidate. Readthrough for Chk2 could alleviate the drug resistance phenotype in cancer.

The SNU-251 ovarian carcinoma cell line carries a nonsense mutation at amino acid residue 1815 of BRCA1 [63]. Lack of 49 C-terminal amino acids as a result of this nonsense mutation resulted in a loss of transcriptional activation of p21 gene – hence cell cycle arrest at G(2)/M could not be sustained. This mutation increased sensitivity to radiation and the chemotherapy agent, Paclitaxel. Introduction of normal allele of BRCA1 to these cells restored chemotherapy resistance. BRCA1 is involved in a significant proportion of heritable susceptibility to mammary and ovarian carcinoma [23]. Clearly again, readthrough here ought to be aimed to be specific and targeted as rescue of BRCA1 nonsense mutations in this context may lead to increased resistance to therapies. This shows that outcome results from varying levels of BRCA1 are context dependent although I have previously considered this factor as a TS that may benefit from readthrough rescue [23].

Four nonsense mutations of p53 TS in mammary gland carcinoma, involving loop domains, L2/L3, correlated with poor response to chemotherapy. Thus certain p53 TS mutations may presage doxorubicin resistance in mammary gland carcinoma. Despite this, during treatment, a partial disease stabilization occurred. This suggests that redundancy exists in regards mutational-drug resistance, that is, other genes appear to compensate for p53 loss. Clearly other, multiple, defects are present within mammary gland carcinoma to result in chemotherapy resistance and these are acting in concert with loss of p53 [64]. Readthrough may still however have some benefit here to restore chemotherapy sensitivity in p53 nonsense mediated mammary carcinoma cases.

In non-small cell lung carcinoma, 10/20 cases presented with p53 mutations. Two of these were nonsense mutations. Cancers with p53 mutations demonstrated an increased resistance to chemotherapy [65]. Clearly, readthrough may have some benefit here to increase the margin of chemotherapeutic sensitivity.

In carcinoma of the upper digestive tract, it was found that oropharyngeal squamous cell carcinomas which were wild-type for TS p53 responded significantly better than mutant p53 cases to radiotherapy. Authors concluded that p53 mutations for carcinomas of the oropharynx lead to an increased radiation resistance with attendant lower survival score [66]. A novel nonsense mutation in p53 was found in this study. Further, it was found that excessive alcohol consumption related to p53 mutation development rather than smoking habit.

Overall, readthrough may have significant benefits for selected cases demonstrating therapy (chemotherapy and/or radiotherapy) resistance in cancer. This section aims to present a novel outlook on the subject of therapy resistance in cancer from the aspect of nonsense mutational alterations. The finding here is that individual contexts must be taken into account before one may consider prescribing readthrough as a

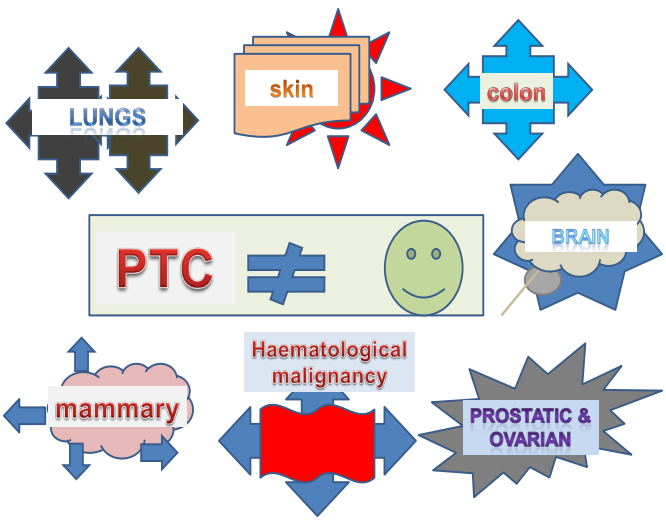

Figure 1: Nonsense mutations (PTC) are relevant within the context of advanced stage cancers, including HM along with a broad range of solid tumours. Arrows/stars represent invasion/spread with higher stage.

pharmaceutical adjunct to standard therapies directed against cancer. Figure 2 outlines the concept of substitution nonsense mutation predisposing to cancer therapy resistance.

Readthrough nonsense targets: Neurological illness

One of the major challenges in medicine for the coming years is to understand illnesses afflicting the brain. With this understanding, improved means to manage such conditions will emerge. At the present time, genetics is revealing the inherent complexity and variation to be found in mental illness. I take the approach of analyzing the relation of nonsense mutations to various conditions with the aim to propose a rescue of these. That in turn may well relate to improving the patient's mental condition. It is a lofty aim to consider the Brain-Mind connection and essentially this is not the topic for my current presentation – yet, having stated this, the molecular basis for many psychiatric conditions is coming further to light. Armed with such information, the approach to mental illness may not be as daunting as originally considered – indeed may be able to be considered with some tangible rational basis.

Low intelligence: Through-out what represents a continuous spectrum of intelligence one finds linked genes presenting with substitution nonsense mutations – from association with intellectual disability to possibly the opposite, viz: prodigy. Some discussion of this field has already been presented by myself [1]. Further examples are presented here. Cereblon is an ion-channel regulator involved in the process of determining higher order, executive cognition. Cereblon forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 and Cullin 4a and regulator of cullins ROC1. This complex ubiquitinates proteins and leads to increased levels of FGF8. FGF8 regulates limb and auditory vesicle formation and is angiogenic. Notably, the drug thalidomide binds cereblon and blocks this particular pathway - consistent with its damaging effects to the unborn. Cereblon also binds to the large conductance calcium-activated potassium channel and regulates this. Neurological disorders occur when this channel is lacking in animal models, such as with cerebellar ataxia and Purkinje cell dysfunction [67]. A model of non-syndromic intellectual disability (NSID) with Cereblon forebrain-specific null mice is proposed to represent an adequate representation of Cereblon alterations on memory and learning for humans [68]. Animal studies show that cereblon is a cytosolic protein with high expression in hippocampus and neocortex. The hippocampus represents part of the limbic system and consolidates information from short-term to long-term memory and is involved in spatial navigation. The hippocampus is essentially part of cerebral cortex and memory loss and disorientation occur when this area is affected in, for example, Alzheimer's disease. Neural plasticity, called long term potentiation, was first located in the hippocampus and is a central mechanism by which memory is stored. The neocortex represents the outer six layers of the cerebral cortex and is involved in higher-order thinking and spatial reasoning and language. It is also involved in determining motor commands and with sensory perception. In the model of human NSID [68] cereblon is portrayed as possessing diverse functionality such as In developmental regulation of ion channels at synapses and orchestrating developmental programs via ubiquitination. In this NSID model, germline cereblon knockout mice (C-/-) were generated and, in addition, specific targeted-deletion of cereblon in forebrain neurons was achieved. In (C-/-) mice, cereblon mRNAs were very low in hippocampus and neocortex and cerebellum. With the targeted forebrain cereblon knockout animals, cerebellar cereblon levels remained intact. The study of these mouse models is consistent with the notion that cereblon may lead to a selective hippocampal-dependent deficit in associative learning and so forms a suitable model for human cereblon aberrations.

The cereblon p.R419x nonsense mutation has been associated with dysregulation of large conductance calcium-activated potassium channel expression in autosomal recessive NSID [69]. As cereblon is a cytosolic protein which regulates the assembly and neuronal surface expression associated with large conductance calcium-activated potassium channels it is not surprising dysregulation may affect brain areas involved in memory and learning. Indeed, the resultant defective channels have a higher intracellular calcium sensitivity, faster activation and slower deactivation kinetics which may explain the cognitive impairments seen in NSID. The p.R419x cereblon PTC mutation results in a protein lacking twenty-four amino acid residues at C-terminus. This altered protein is still able to retain ability for assemblage into Cullin E3 ligase complex and catalyze ubiquitination of cereblon target proteins. This may explain why the intellectual disability phenotype is 'mild'. Nevertheless, this partly C-terminally truncated protein demonstrates increased levels of auto-ubiquitination and is thus more readily degraded by the proteasome apparatus.

More recently, an investigation was performed aimed to rescue cereblon p.R419x nonsense-mediated NSID via proteasomal inhibition [70]. The proteasomal inhibitor: Bortezomib, was used - an accepted agent in the management of several haematologic malignancies (viz: relapsed multiple myeloma and mantle cell lymphoma). Its usefulness is probably at least in part due to inhibition of degradation of pro-apoptotic factors. Adverse reactions include a peripheral neuropathy and a mild myelosuppression. Secondary shingles is an issue although preventable in part via co-administered acyclovir. Gastrointestinal mal-effects are more commonly found. Bortezomib was found to restore levels of cereblon from the p.R419x mutation *in vitro*. The suggestion here then is that bortezomib could offer potential therapeutic treatment for cereblon deficient cells, for example, those presenting with the key nonsense mutation: p.R419x. As the R419X mutation is present in the last exon of cereblon it is not expected to trigger reactive nonsense mediated decay (NMD). The truncated protein is translated

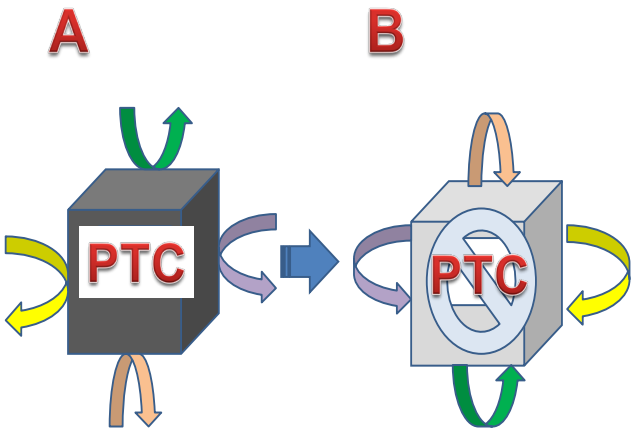

Figure 2: Nonsense mutations may relate to cancer therapy resistance in selected scenarios. Boxes represent tumour and rounded arrows represent in: (A) resistance to various therapies; (B) arrows are reversed to indicate increased sensitivity to therapies when relevant PTC are rescued by read through.

in cells and is capable of forming E3 ligase and promoting appropriate ubiquitination of various physiological targets. Proteasomal degradation of the altered truncated cereblon protein appears to be the mechanism behind its lower levels within cells and hence results in failure to recoup its appropriate functionality. Although proteasomal inhibition was indicated to be a means to augment cereblon within the cell the overall question remains: shall restoration of cereblon levels in patients result in improved cognitive activity? The issue is that cereblon deficiency during development could result in irretrievable neurological damage that cannot be readily restored. However, having said this, animal models suggest that neurocognitive phenotypes may be reversed in the adult state – thus bringing hope to countless millions of people afflicted with neurological developmental disorders. These findings included animal models of autism, learning deficits in tuberous sclerosis and a wide range of neurodevelopmental disorders [71,72]. Clearly, these results have a major impact in considering readthrough as a 'rescue' approach in that neurological deficits appear to be able to be restored in the adult, viz: post-developmental state. It is obvious that if one may restore cereblon activity and affect a physiological response in post-development phase then readthrough therapy, with such safely used agents as Ataluren [23] and Amlexanox [1] ought to be highly effective for the p.R419x mutation, and therefore, intuitively, for associated NSID.

Fragile X intellectual disability is due to a mutation that removes activity of the fragile X mental retardation protein (FMRP). In a fragile X retardation gene knockout mouse, an expression vector that produces the brain isoform of FMRP was shown to rescue enhanced long-term depression, a type of plasticity that is linked to cognitive impairments in Fragile X syndrome. Hippocampal injections of expression vector restored enhanced long-term depression activity to wild-type level. Clearly, here too, post-developmental protein replacement or rescue therapy may potentially significantly improve cognitive functionality [73].

The genetics of autosomal recessive NSID may be certainly more complex in that with another study three correlating genes to the condition were found. Mutations in these NSID-related genes were truncating-type nonsense alterations [74]. Such changes were consistent with a severe loss of functionality and indeed that as authors stated: 'The future objective will be the development of diagnostic kits for molecular diagnosis in mentally retarded individuals in order to offer at-risk families pre-natal diagnosis to detect affecting offspring'. The hope being here in diagnostics as well as of course, future directed therapies at rescuing such mutations – as I propose in this article, with the use of readthrough with readily available, apparently safe agents such as Amlexanox. Prevention and correction being a theme throughout my current presentation.

A more recent study suggests that apart from X-linked mental retardation, autosomal recessive NSID is very heterogeneous with ten genes to date being established for this illness – understandably this condition ranks as a heavy burden on society [75]. A key point to remember though is that as many genes/mutations have been implicated in NSID, already in a number of cases as indicated above, a commonality may be seen amongst these changes. This rests with the alterations involving PTCs. Again, it must be recalled that readthrough presents an advance over other approaches in that it targets in a mutation-specific fashion rather than in a gene-specific manner.

Further examples of the complex genotype/phenotype variation that is seen in the relatively common autosomal recessive NSID include studies with TRAPPC9 (Trafficking Protein Particle Complex subunit 9) [76]. Nonsense mutations in TRAPPC9 have been found within five large Middle Eastern families and linked to NSID. A further pathogenic mutation in TRAPPC9 was found in two Italian cases consisting of a homozygous splice site mutation resulting in exon skipping with frameshifting and PTC – hence resulting in loss of functionality. Trafficking Protein Particle Complex subunit 9 plays a role in NF-kappaB signaling. NF-kappaB, an ubiquitously expressed transcriptional factor dimer, regulates innate and adaptive immunity along with cellular proliferation, apoptosis and development (see Section: Amlexanox and innate immunity). Both canonical (standard) and noncanonical kinase pathways mediate activation of NF-kappaB. Canonical pathway mediates via activation of IkB kinase (IKK) complex of two subunits viz: alpha and beta and a regulator subunit, NEMO. On activation, the complex phosphorylates IkB inhibitor to NF-kappaB leading to ubiquitination and degradation via the proteasome and releases NF-kappaB dimers which in turn, migrate to the nucleus and activate an arrary of survival genes. The noncanonical pathway activates NF-kappaB via another pathway - hence the term 'non canonical' - non standard [77]. This involves TBK1 and ikkepsilon kinases (see Section: Amlexanox and innate immunity) [78]. TRAPPC9 is an NF-kappaB inducing kinase and ikappaB kinase (ikk-beta) binding protein. As such it is considered to represent an activator of NF-kappaB through increased phosphorylation of the IKK complex. In intellectual disability it has been shown via microarray expression analysis and linkage mapping that an NF-kappaB signaling defect may be present [79]. Autosomal recessive inheritance represents ~25% of NSID and is genetically very heterogeneous. A Tunisian family of three children with NSID delineated the TRAPPC9 gene as a candidate NSID causative gene. The germline substitution nonsense mutation, p.R570x in exon 9 was considered to be the culprit alteration involved. TNFalpha is an activator of NF-kappaB and in the context of the p.R570x mutation, this cytokine activation pathway is disrupted and so NF-kappaB signaling is altered [79]. Altered NF-kappaB signaling is broadly involved in neuropathology, for example, in chronic neurological disorders such as Alzheimer's and Parkinson's and Huntington's diseases. NF-kappaB is involved in synaptic plasticity and long term memory and inhibition of NF-kappaB blocks long term potentiation in hippocampus and amygdala. So NF-kappaB is essential for long term memory formation in the hippocampus in particular. Thus it may come as little surprise that NF-kappaB is involved too with intellectual disability. Certainly this expands the pathophysiology of such neurological diseases and provides a mechanism for therapeutic approach – viz: use of readthrough as a preventive/management strategy in nonsense mediated NSID via the NF-kappaB axis. Amlexanox has effects unrelated to readthrough that target noncanonical kinase pathway of NF-kappaB and these need to also be taken into consideration in this discussion (see Section: Amlexanox and innate immunity). Other agents, such as Ataluren, may however be used for readthrough rescue that do not apparently have the attending complication of direct NF-kappaB interference.

De novo mutations in synapse-related genes are present in a significant percent of sporadic NSID [80]. Three nonsense mutations in 95 cases of sporadic NSID – pathogenic truncating/splicing mutations - were found in SYNGAP1, STXBP1, SHANK3. These were not found in controls. SYNGAP1, STXBP1, SHANK3 are involved with glutamate receptor physiology. SYNGAP1 is a Ras GTPase-activating protein required for development of cognition and synapse formation. STXBP1 is syntaxin binding-protein-1 involved in vesicle trafficking and neurotransmitter release. SHANK3, SH3 and multiple-ankyrin

repeat domains-3, plays a role in synapse formation and dendritic spine formation. Disruption of SYNGAP1 protein is related to a relatively common cause of NSID [81]. In 94 persons with NSID, SYNGAP1 was sequenced and two substitution nonsense mutations, viz: p.K138x and p.R579x were found in patients - not in controls. This is consistent and again, supportive, of the importance of synapse functionality in NSID.

In a patient with NSID and autism a de novo nonsense mutation, p.R525x, was found within the forkhead DNA-binding domain of FOXP1. This factor FOXP1 (forkhead box protein P1) is a member of the family of FOX transcriptional factors involved in developmental regulation. The patient with the nonsense mutational disruption showed language impairment, mood lability and aggressiveness with obsession-compulsion disorder [82].

X-linked mental retardation has a nonsense mutational basis too. CUL4B gene alterations appear in syndromic X-linked ID where Cullin 4b is a member of the cullin protein family and forms a complex that functions as an E3 ubiquitin ligase and catalyzes polyubiquitination of protein substrates. In a family with three affected brothers a novel nonsense mutation, p.K703x, in exon 18 of CUL4B was determined. This mutation is passed from the asymptomatic mother since Cul4B is located at Xq23. Speech impairment with tremor/seizures suggests varying cerebellar effects [83].

Intellectual disability is a classic situation reflecting the subtlety of the human phenotype hence the difficulty of modeling this condition which is due to the vast genetic variation. This poses clearly a major challenge to appreciate illnesses particularly of the neurological system. Fortunately, as stated, we can work from reverse genetics today to help avoid confusing phenotypic subtleties and define a phenotype from a particular genotype [1], that is, in a 'reverse genetics' fashion. By offering mutation-specific therapies such as readthrough which are not gene-dependent – rather, genotype-specific – one may, in turn, be able to come to the clinician's aid in managing complex human neurological illnesses.

Doubtlessly, Amlexanox with its NMD inhibitory and readthrough capacity [1] ought to be trialed for ID – since, as discussed above, post-developmental correction of protein function is possible. Results would be predicted to be rather dramatic and any improvement in cognitive ability would be most welcome by many individuals Worldwide.

Depression and mental illnesses: Schizophrenia and bipolar disorder show a high degree of heritability with ~80% cases showing a genetic component. Indeed, Genome Wide Association Studies (GWAS) suggest an overlap of genetic risk between these illnesses. So it comes as little surprise that genetic variants within a candidate gene, ABCA13, show overlap amongst the neurological conditions: schizophrenia, bipolar disorder and depression [84]. The lipid transporter, ABCA13, is thus a susceptibility factor for both schizophrenia and bipolar disorder. Substitution nonsense alteration in this gene was isolated in a case of schizophrenia. This mutation and other variants showed a significant risk for schizophrenia development with bipolar (manic-depressive disorder) and also major depression. The nonsense mutation, p.R4728x, is noted to segregate with bipolar disorder and depression in two families and in two other families the mutation segregated with schizophrenia and also depression. ABCA13 belongs to the superfamily of ATP-binding cassette family of transporters. Another family member of note is ABCC6. Mutations in this protein lead to Pseudoxanthoma Elasticum [1]. The nonsense alteration, p.R4728x, occurs in the A loop of ABCA13 thus suggesting that this is a key region for activity. Location-wise, a similar mutation occurs in the conserved A loop motif in human ABCC6 viz: p.R1275x, leading to Pseudoxanthoma Elasticum. As Pseudoexanthoma Elasticum has already been used as a model for readthrough [1] of the superfamily of ATP-binding cassette family of transporters then it is imaginable that readthrough for psychiatric illness could be equally achieved. Overall, disruptions of the lipid membrane shuttle mechanism involved in vesicular trafficking, signal transduction and transcriptional regulation along with abnormalities in lipid metabolism have all been strongly correlated with psychiatric illness. Membrane phospholipids and expression of factors required in sphingolipid metabolism are altered in brain tissues of schizophrenics and are upregulated in response to antidepressants. Readthrough, by rescuing pathogenic nonsense codons in the ATP family of transporters could definitely find a central place within the psychiatric therapeutic armamentarium.

Other neurological conditions: Aggression and obsession-compulsion: It is interesting to probe whether various mood disorders and behavioural states may be related to nonsense mutations. Aggressive personality-types are difficult to classify. In some instances though, there appears a definite and tangible genetic component. It has previously been noted that a nonsense mutation in the X-linked monoamine oxidase A gene is linked to aggressive behaviour [85]. Brunner syndrome is caused by a monoamine oxidase A deficiency leading to excess monoamines in the brain such as serotonin and dopamine and noradrenaline. Intellectual disorder and aggressive behaviours characterize the illness. In a large family several affected persons showed a substitution nonsense mutation in exon 8 of MAO A gene [86]. The condition is X-linked [87] and, as such, typically affects males. Animal models are useful in leading to an improved defining of this illness. Monoamine oxidase A knockout mice have been produced containing a substitution nonsense mutation in exon 8 analogous to that occurring in the human condition, Brunner syndrome. These mice display increased aggressive behaviour to intruder mice [88]. Clearly, this model would form an excellent test of readthrough for nonsense mutational rescue to correct such a severe behavioural handicap.

Interestingly, at the other end of the spectrum, an individual with a noted jovial and agreeable personality was found to carry a de novo germline nonsense mutation, p.R239x, in exon 6 of SATB2 gene [89]. Dominant negative effects were considered to result in the phenotype as mutant RNA was not subjected to NMD and protein dimerization was intact. SARB2 protein comes from a family encoding nuclear matrix-attachment region proteins. This patient also exhibited features more typical of a syndrome viz: cleft palate and osteoporosis, intellectual disability and epilepsy in addition to his sparkling outlook. Overall, the nonsense mutational alteration may be considered to be a significant disease-related event that could well benefit from 'rescue'.

In myoclonus-dystonia syndrome (MDS) an autosomal dominant pattern of inheritance is observed [90]. The disease is characterized by myoclonic and dystonic muscle contractures and psychiatric problems, such as obsessive-compulsive disorder, depression and anxiety. The phenotype relates to a novel truncating mutation within exon 4 of the epsilon-Sarcoglycan gene, SGCE. Most obsessive–compulsion disorder individuals present independent of physical problems and so further analysis is needed to examine whether nonsense mutations are present in other genes relating specifically to this psychiatric complex.

Tourette's - Talent and tics – a neurological issue: A nonsense mutation has been found in a family with Tourette's syndrome [91] with an autosomal dominant inheritance pattern. Tourette syndrome

is a neurodevelopmental disorder characterized by chronic motor and vocal tics. It remains a heterogeneous genetic condition. A heterozygous mutation, p.W317x, was found in exon 9 of HDC gene (histidine decarboxylase), an enzyme that produces histamine from histidine. The truncated protein is missing key portions of the active domain of this enzyme. It is considered that the truncation would lead to haploinsufficiency and thus decreased levels of histamine in the brain. Dominant negative action with the truncated product is also entirely feasible and evidence has been produced along those lines [91]. The authors suggest that therapies aimed to moderate deficiencies in histaminergic neurotransmission shall open up avenues for treatments of Tourette's syndrome.

Having said this, Tourette's syndrome has been suggested to have been a diagnosis for certain very gifted persons [92]. Perhaps nonsense mutations may lead to some increased mental capacity – more information may certainly be needed to establish this enticing conclusion.

Other considerations relating to neurological conditions: It will be interesting to investigate if nonsense mutations play any significant role in substance addictions – this could be a fruitful area of exploration for studies in the future - in order to develop readthrough targets in this, a most relevant area of contemporary psychiatry. Defining the genetics behind serious psychiatric illnesses leading to sociopathic behaviours as well as the more subtle, yet disturbing and handicapping broad spectrum of personality disorders remains a vast challenge. Many of these conditions may well have a very heterogeneous genetic make-up, again making for all the more challenge at arriving to novel therapies. As readthrough is mutation-specific rather than specifically gene-targeted some hope may be available along the lines of development of universal readthrough therapeutics for psychiatry.

A key matter of importance rests with negotiating the blood brain barrier, as I have outlined earlier in regards readthrough agents [1]. This shall have to be assessed on an individual basis for all the various generational readthrough drugs that are currently available.

My proposition for the use of Amlexanox as a readthrough agent with NMD inhibitory capacity is certainly novel in terms of targeting a broad spectrum of neurodegenerative/psychiatric conditions (Figure 3). This present detailed investigation of neurological illness highlights involvement of the NF-kappaB signal axis and reveals that Amlexanox needs to be nonetheless cautiously trialed by neuroscience researchers to gauge its appropriate effects in the context of neurological diseases since it modulates NF-kappaB activity.

Bone disease and nonsense mutations

Osteogenesis imperfecta and osteoporosis: Bone diseases are many and varied. From cancer both as primary and metastatic disease to degenerative conditions and age-related illnesses and developmental disorders, the bony skeleton reflects to a large degree overall health and functional well being. One of the components mutated in bone disorders involves the Wnt pathway including Wnt1. Wnt1 is a proto-oncogene protein encoded by WNT1 gene. The WNT family is involved in signaling functions with Wnt1 being highly conserved and is significantly involved in development. Overall, the Wnt signaling pathway concerns itself with signal transduction through interaction of Wnt ligand with Frizzled family receptor. Although certainly involved in cancer (as a prosurvival factor [39]) Wnt signaling has a broad application in biology from cell fate determination to developmental cellular migration. It therefore has a major 'say' towards appropriate formation of tissues such as bone, heart and muscle. Consistent with these mechanisms, a WNT1 nonsense mutation was detected in a family of two children with recessive osteogenesis imperfecta – a congenital brittle bone disorder [93]. A homozygous nonsense mutation, p.Ser295x, was determined for WNT1. This leads to an impaired WNT1 protein which cannot induce canonical WNT signaling. This in turn leads to problems with mineralization as WNT1, from animal models, is seen to be expressed in bone marrow and in haematopoietic progenitor cells along with a subset of osteocytes. WNT exercises a cross-talk between these cell types and defects in this signal relay system could disturb appropriate bone formation.

Other families affected by bone fragility have been noted to carry WNT1 substitution nonsense mutations that may initiate NMD [94]. It has been indicated that mutational variants of WNT1 may predispose to low-bone-mass phenotypes. Understanding this involvement may enhance developments of novel, more effective therapies, not only for congenital fragile bone disease but also for the relatively common age-related disease: osteoporosis [95]. Again, this tends to support the contention of viewing ageing as a disease – in a number of age-related conditions nonsense mutations are certainly to be found [1]. Bone disease is no exception to this, as in early onset osteoporosis mutations in WNT1 may be found [93,95,96].

An essential hallmark for osteoporosis is low bone mineral density (LBMD). GWAS - genome wide association studies - of LBMD determined a nonsense mutation in the leucine-rich-repeat-containing G-protein coupled receptor 4 (LGR4) gene. The mutation led to the production of a stop codon at position 126 and completely disrupted protein function [97]. LGR4 potentiates the canonical WNT signaling pathway and acts too as a negative regulator of innate immunity via inhibiting TLR2/4 and proinflammatory cytokine production [98]. Therefore it is not surprising that LGR4 is associated with LBMD and osteoporotic fractures due to its close association with WNT signaling. The LGR4 nonsense mutation has a multifunctional capacity in Icelandic individuals who were screened – these being increased risk for skin squamous cell carcinoma and biliary tract cancer. The phenotype of carriers of the mutation overlaps with that of the LGR4 mutant mouse and therefore the animal model would appear ideal for

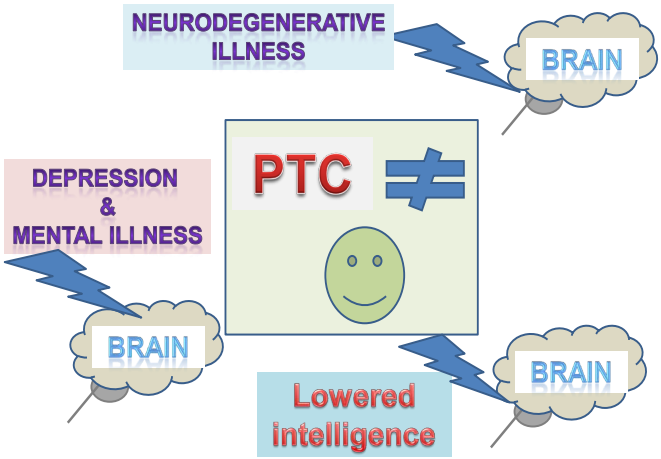

Figure 3: Nonsense mutations relate to various key central nervous system conditions such as mental illnesses, intelligence as well as neurodegenerative disease.

testing readthrough as a basis for management of the cognate human disease process [97]. This would certainly appear attractive in terms of formulating readthrough for osteoporosis therapy.

Rickets: In rickets there is dysfunction of bone formation due to inappropriate regulation of vitamin D, phosphorus or calcium. This in turn may lead to fractures and deformity and is a particularly prevalent paediatric condition in many countries. Hereditary vitamin D-resistant rickets is an autosomal recessive condition caused by inactivating mutations in vitamin D receptor gene. Vitamin D regulates bone homeostasis and mediates its actions via interacting with the vitamin D receptor, a nuclear transcription factor. A homozygous C to T transition in exon 2 of vitamin D receptor resulting in p.R50x was determined in two affected children from the one family [99]. In another study, the same mutation, p.R50x, disrupts the Vitamin D receptor [100]. This was seen in two unrelated patients with severe early onset rickets with hypocalcaemia, hypophosphataemia and scant hair. Another mutation, this time p.Q317x, disrupting the ligand binding domain of the vitamin D receptor has been found in a patient with vitamin D resistant rickets [101]. This truncation deletes 110 amino acids of the ligand binding domain of the receptor and results in loss of 1,25-dihydroxyvitamin D binding with its target.

In X-linked hypophosphataemic rickets there is a phosphate wasting phenotype with decreased renal phosphate reabsorption. The PHEX gene is involved which is a transmembrane endopeptidase controlling bone and dentine mineralization and renal phosphate reabsorption. X-linked rickets presents with bony deformities and pain and hearing loss. Of relevance is a novel nonsense mutation of PHEX, viz: p.Y245x [102]. Appropriate treatment to date has been medical via phosphate supplements and 1,25-dihydroxyvitamin D. Another addition to the treatment armamentarium would be with the use of readthrough – as I propose in this article.

Osteodystrophy: This is an all-encompassing term denoting dystrophic bone growth. In a case of Albright hereditary osteodystrophy (AHO) - also called Pseudohypoparathyroidism type 1a - there is a lack of responsiveness to parathyroid hormone. This results in low serum calcium, high serum phosphate and also high serum parathyroid hormone. Affected individuals have short stature and mild intellectual disability. Seizures may also occur due to the hypocalcaemia. GNAS1 (the stimulatory G-protein alpha subunit – a factor involved in signal transduction) shows a nonsense mutation of exon 13 - codon 384 [103]. As expression of GNAS is regulated by imprinting then it is tempting to speculate that a methyl-dependent deamination reaction may have produced this nonsense mutation [1].

Progressive osseous heteroplasia - POH – a condition showing dermal ossification and bone formation in deep muscle and fascia along with AHO was seen in two unrelated persons. Mutation p.Q12x is seen in the GNAS1 gene in the milder affected patient. This relates GNAS1 deficiency to extra-skeletal ossification [104].

Readthrough may have potential significant benefits for those with nonsense-mediated osteodystrophies.

Paget's disease: Paget's disease is a condition with focal areas of increased bone resorption and increased osteoclastic activity. Nonsense mutation variants are observed in the SQSTM1 gene (sequestosome-1) [105]. These tend to cluster around the ubiquitin-associated domain. There is a link between SQSTM1 and Autophagy-linked FYVE-domain-containing protein (ALFY) in osteoclast cells. When osteoclasts are starved, to induce autophagy under external challenge, the SQSTM1-ALFY complex relocates from nucleus to cytoplasm. Mutations alter the response to external challenge leading to pathology or excessive osteoclastic activation/dysregulation. Indeed, it would be interesting to examine whether readthrough may make a significant clinical impact on management/reversal of the signs/symptoms of nonsense mediated Paget's disease.

Dentinogenesis: The proper formation of the teeth is related to appropriate orchestration of the mineralization process. A nonsense mutation, p.Gln45x, in exon 3 of Dentin Sialophosphoprotein (DSPP) in a Chinese family with dentinogensis imperfecta Shields type II was found where there was discolouration and severe attrition of the teeth and obliterated pulpal chambers [106]. Collagen disorders too are reflected in tooth-related disturbances. For example, a homozygous nonsense mutation in ADAMTS2 (p.Trp795x) results in type VIIC Ehlers-Danlos syndrome with agenesis of multiple teeth along with focal dysplastic dentin defects of remaining teeth [107]. ADAMTS2 is a procollagen proteinase, responsible for processing various procollagens and is required for collagen maturation. Not surprisingly, it is involved in the appropriate formation of mineralized tissue. Readthrough may indeed also find a place within the dental armamentarium as I have earlier on intimated [1] for nonsense-mediated amelogenesis imperfecta-gingival hyperplasia syndrome management.

Scoliosis: This term derives from the classical Greek: 'skoliosis' meaning bending or obliquity. In this condition the patient's spine is curved in a complex three-dimensional type of deformity of the 'joints' or disks of the spine. The disease may present at birth (congenital) or idiopathically – that is, presenting either in early growth phase or later on into adulthood. Secondary scoliosis to spinal muscular atrophy and cerebral palsy may also be observed as part of a broader syndrome complex. The most frequent form of the condition is that of late onset idiopathic type and is self limiting.

ALMS1 gene mutations have been linked to Alström syndrome - a condition with decreased visual abilities and obesity and insulin resistance with diabetes type II along with other multiple organ defects. Commonly, nonsense/truncation mutations are found in this gene which co-segregate with this genetic disease [108]. Clearly, readthrough could well have a benefit here if commenced early in development and carried on throughout life. Additionally, the insulin resistance regulating capacity of Amlexanox, the selected readthrough agent for the present article, would in addition prove of benefit for these Alström sufferers (v.i.).

In Desbuquois dysplasia presents with short stature and scoliosis - and among seven mutations within the Calcium-Activated Nucleotidase 1 gene (CANT1) [109] – four produced premature truncation of the gene product. This nucleotidase is presumed to be intimately involved in endochondral ossification. Perhaps readthrough may produce benefits in manageing this condition – turning CANT1 into CAN!

Many other genes are involved in syndromes related to scoliosis and doubtlessly, over time, further genetic exploration shall reveal that idiopathic varieties of scoliosis have a defined genetic basis – perhaps also lending themselves to readthrough rescue from an early age.

Joint disease: No discussion of bony disease would be complete without noting arthritic conditions.

Osteoarthritis is a major cause of morbidity in the community, particularly the ageing population, and is the most common reason for

hip and other joint replacements. Osteoarthritis is due to a variety of causes such as hereditary predisposition along with developmental and metabolic disease, all culminating in the loss of joint cartilage. SMAD3 mutations have been found in families with syndromic osteoarthritis. SMAD3 is a TGF-beta family member involved in cell signaling. Two frame-shift mutations within important regions of SMAD3 led to truncated transcripts - presumably subject to NMD [110,111]. These mutations probably cause loss-of-function with TGF-beta signals not being correcting propagated via SMAD3. Vascular anomalies are also evident – relating to the phenotype. Osteoarthritis syndrome is an autosomal dominant form of thoracic aneurysms/dissection and early onset osteoarthritis is due to such SMAD3 gene mutation. Again, it may model a premature form of ageing – taking ageing as a 'disease' - and is consistent with nonsense mutations being involved in early onset ageing, as I have mentioned earlier [1].

Spondyloepiphyseal dysplasia tarda (SEDT) is a genetically mixed disorder associated with early-onset osteoarthrosis. A recessive form has been determined in one family. A novel nonsense mutation in the SEDL (Sedlin) gene was found and the message here is that SEDT should be considered in early-onset osteoarthrosis/premature ageing [112]. Evidently, SEDT represents a particular subtype of early-onset ageing of the skeleton – nonsense mutations being involved in a number of these cases.

A further discussion of arthritis from an inflammatory viewpoint is pursued later (v.i). Amlexanox can be seen as possessing a dual quality of readthrough with NMD inhibitory capacity as well as an ability for modulation of inflammatory response - both of these aspects could be beneficial for arthritic condition management/prevention.

Overall, this detailed account of nonsense mutations and attendant NMD in relation to bony diseases reveals how my proposed novel application of Amlexanox to such conditions could well be most beneficial (Figure 4).

Nonsense mutations and bleeding disorders

Von Willebrand disease is the most common hereditary coagulation abnormality and is due to lack of functional von Willebrand factor (VWF) - a multimeric protein required for platelet adhesion. Von Willebrand's disease type 3 is autosomal recessive for mutation of VWF gene and just under half of the alleles outlined in a Finnish study showed a c.2435delC frameshift mutation, oftentimes found in the Baltic sea area. A substitution nonsense mutation, p.R1659x, is found in 40% of alleles examined. Authors conclude that two main mutations viz: c.2435delC and p.R1659x result in the majority of type 3 von Willebrand disease in Finland [113]. As these mutations can produce PTC events and potentially also lead to NMD of the VWF then readthrough can be seen to be an effective means to prevent and manage nonsense-mediated von Willebrand disease.

In a large VWD type 3 pedigree, PTC has arisen as a result of insertion mutation c.7674-7675insC in VWF exon 45. Heterozygote carriers of the mutation possessed lower than normal VWF mRNA levels as the mutation allele has undergone NMD [114]. Hence, NMD plays a role in pathogenesis of VWD via quantitative deficiency of VWF - a substitution nonsense mutation does not have to be directly involved to result in NMD.

Readthrough and combined NMD inhibition via the same drug (viz: Amlexanox [1]) could well prove to be most efficacious in the clinical management of VWD.

Fibrinogen disorders result in delayed plasma clotting. Nonsense mutations have been found in ~7% of fibrinogen genes within an Indian population [115] - again pointing out the potential of readthrough for another bleeding diathesis.

Factor VIII mutations dictate a phenotype of haemophilia A. Patients with null mutations show their first general bleed and joint bleed (haemarthrosis) at younger median age as compared to patients not carrying null mutations [116]. Fifteen nonsense mutations of Factor VIII in an Iranian population have been categorized as novel. This reflects the large degree of genetic heterogeneity in Factor VIII mutations overall as well as the potential for such cases to form excellent readthrough targets [117].

In haemophilia B the Factor IX genotype determines to a large degree clinical phenotype. In 52 individuals 7 cases presented with nonsense mutations and were associated with higher risk as compared to missense alterations [118]. Clearly, here too, readthrough may well have a significant place in the medical management of nonsense-mediated Factor IX disease. Interestingly, spontaneous ribosomal readthrough may occur and is demonstrated in Factor IX gene [119]. There were appreciable levels of secreted Factor IX with mutations: p.R162x (~5% of wild-type); p.R294x (~3% of wild-type); p.R298x (~2.5% of wild-type). The mutant: p.L103x, shows negligible production of extended product. Although the pattern of readthrough by Western blotting appeared to represent mainly truncated forms, there were, for p.R294x and p.R298x, traces of full length Factor IX. This was not apparent for p.L103x, which appeared to trigger NMD. Artificially enhanced readthrough via drug therapy that also inhibits NMD could be predicted to significantly augment native readthrough and result in qualitatively improved functional product: Factor IX.

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive primary immunodeficiency with mutations in WAS protein – WASP. This protein is involved in cell signaling, translating external signals from cell surface receptors to the cell's actin cytoskeleton. WAS syndrome shows thrombocytopenia with infections, eczema, autoimmune disease and haematologic malignancies. A novel nonsense mutation – p.Q19x, was found in a syndrome case. The patient developed allergy manifesting as angioedema with urticaria and had concurrent cytomegalovirus infection managed by long term ganciclovir [120]. It is tempting to

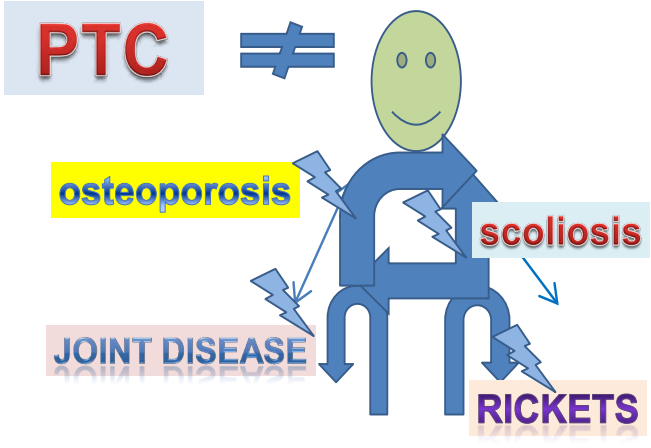

Figure 4: Bone and joint disease may relate to nonsense mutations.

suggest that readthrough could prove to be a most useful addition to the armamentarium of therapies for nonsense-mediated WAS.

Interestingly, converse to the situation with bleeding diatheses, conditions leading to excessive coagulation are important as well. Factors involved here include: antithrombin, an anticoagulant whose deficiency leads to risk of venous thrombosis and pulmonary embolism as antithrombin inactivates various enzymes of the coagulation system; Protein C is an anticoagulant factor along with Protein S. Nonsense mutational alterations (at a prevalence of ~9%) in antithrombin/Protein C/Protein S have been linked to excessive pro-coagulation disorders [121]. Readthrough can be seen to also have a potential impact in management of these clinically significant coagulation disorders.

Overall, this analysis of the literature with respect to disorders of coagulation serves to highlight and reveal the importance of truncating mutations in this context. The novelty of using Amlexanox as a readthrough agent in coagulopathy states is a focus of my present thesis (Figure 5).

The five senses

A convenient outline of the impact of nonsense mutations on human health may be appreciated best from an analysis of how such alterations may impact on our key vital senses.

Eye and ear: I have already outlined studies examining the importance of PTCs for eye diseases as well as proof of principle that readthrough may offer a useful remedial method of approach to various genetic eye diseases such as retinitis pigmentosa and Usher's disease [1]. Protein truncating mutations in ZEB1 cause PPCD3 – posterior corneal dystrophy – this supporting a clear cut genotype/phenotype correlation [122]. ZEB1 is a zinc finger E-box-binding homeobox 1 factor and posterior polymorphous corneal dystrophy leads to decreased vision due to corneal oedema. Many other genes involved with PTCs correlate to eye diseases – as eye diseases affect vision for all its varied levels from clarity to colour – and are many and complex genetic conditions. In another example, [123], Leber congenital amaurosis (LCA), is a severe retinal degeneration and common cause of blindness in children – thus far incurable. Several LCA genes have been identified. Through screening a group of 852 patients affected by LCA three RD3 mutations have been found in seven unrelated LCA families – two of these were nonsense mutations predicted to result in loss of function. Five families from the southern Mediterranean region had the same mutation viz: p.R38x – consistent with an 'ancient founder' effect. The RD3 gene is a 23 kDa protein involved in expression of guanylate cyclase in photoreceptor cells. Adeno-associated viral vector transfer of RD3 gene protein restores guanylate cyclase physiology in photoreceptor cells in a mouse model for LCA [124]. This then rests as a 'proof of concept' that restoration of this protein may be beneficial and offers considerable hope for readthrough as a viable therapy for the oftentimes determined, nonsense mediated LCA. A case has been outlined with extensive follow-up of LCA carrying novel compounded heterozygous mutations in the RPE65 gene (retinal pigment epithelium-specific 65 Kda protein – which is involved in conversion of all-trans retinol to 11-cis retinal in phototransduction for visual pigment regeneration in photoreceptors). A maternal allele, p.K303x, and paternal allele, p.Y431C, missense alteration were found. There were severe visual defects and absence of rod and cone electroretinogram response. Early childhood exam showed normal colour recognition and visual acuity of 20/60 for both eyes. But 30 years on the acuity decreased to 1-2/200 bilaterally - so progressive visual loss occurred during life, which must have been very debilitating [125].

Another instance of eye disease that is prevalent and may lead to problems with career and in daily existence is that commonly called: colour blindness. The most common cause is a fault in development of various sets of retinal cones which perceive light colour. Many of the genes involved in colour vision are located on the X-chromosome and as such colour blindness is much more frequent in males. Progressive cone dystrophy carries mutation in CNGB3 [126]. Absent cone response and normal rod response on electroretinography and colour blindness and poor visual acuity from early age is seen in a patient homozygous for CNGB3 mutation Thr383fs generating a PTC. Other family members had this mutation in one allele and a missense mutation with the other allele in the pore domain of this, the cone CNG cation channel beta-subunit protein.

In a Mid-Eastern family from The Palestine, autosomal recessive retinal degeneration has been noted [127]. A novel homozygous nonsense change viz: p.Gln461x, in exon 13 of the CDHR1 gene presents. The retinal degeneration paralleled night blindness and progressive acuity loss and cone loss from early age. CDHR1 is Cadherin-related family member 1 – a member of calcium-dependent cell adhesion factors.

A novel homozygous nonsense mutation affecting CABP4 in two children of the same family caused a phenotype of reduced cones and mildly reduced rods as inferred via electroretinography – night blindness was not a feature. The mutation is p.Arg216x in CABP4 and NMD was not a feature by RT-PCR. The overall phenotype was that of reduced visual acuity and abnormal colour vision [128]. CABP4 - Calcium binding protein 4 – is involved in synaptic function via regulation of calcium influx and neurotransmitter release in photoreceptor synaptic termini.

Oftentimes one finds a link between ear and eye defects, such as with Usher's [1]. Other instances are those involving a myopia and deafness [129]. In this syndrome, myopia (shortsightedness) and sensorineural deafness are features. Three families investigated demonstrated three homozygous nonsense mutations (p.R181x, p.S297x and p.Q414x)

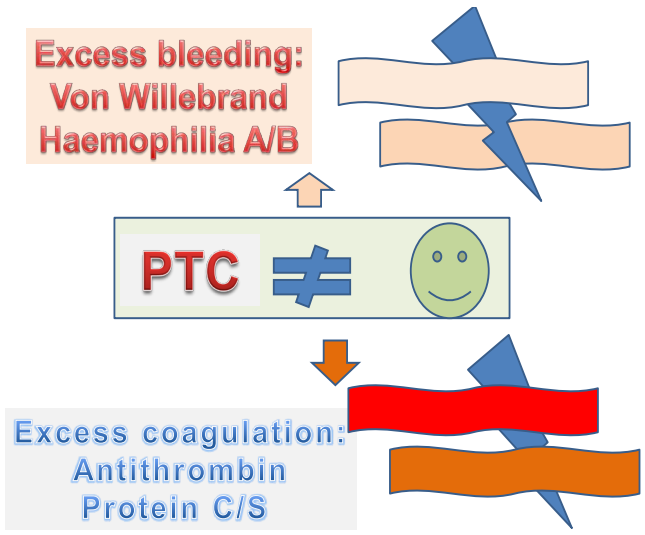

Figure 5: Nonsense mutations and disorders of coagulation.

in SLITRK6 - a leucine-rich repeat domain transmembrane protein. These mutated proteins all had defective cell surface localization and the factor is presumably involved in eye and hearing formation. Readthrough may well find a place in rescuing such a defect based on these genetics.

Examples exist of nonsense alterations resulting in hearing impairments alone. For example, nonsense mutations p.Gln520x and p.Arg925x are present in the Otogelin-like gene in paediatric examples showing significant deafness [130] - although, admittedly, hearing impairment itself is very genetically heterogeneous. Of eight novel mutational variants in the SLC26A4 gene - sodium-independent chloride/iodide transporter - a nonsense mutation was found viz: p.W472x [131]. This was in a nonsyndromic hearing loss case - the most common form of neursensory deafness with a large diversity of genetic types.

Certainly hearing loss is a common sensory disability and in ~2/1000 children, the gene GJB2 for connexin 26 (gap junction beta=2 protein) has defects leading to the most common form of congenital deafness in the developed World. Two Portuguese persons with nonsyndromic sensorineural moderate/severe hearing loss presented with a new mutation viz: p.Leu213x in the GJB2 gene. These cases are compound heterozygotes with the other allele being a deletion for GJB2 [132].

A matter of Taste: In a person presenting with noted difficulty in taste as well as for aroma sensitivity [133] an altered ARG1 gene allele with a p.K75x mutation led to argininaemia. Some neurodevelopmental delay was also seen. ARG1 makes arginase which controls a final step in the urea cycle – mutation leads to an increase in arginine and ammonia in the serum.

A nonsense mutation, p.R238x, in the bovine FMO3 ortholog results in a rather fishy off-flavour for cow's milk. This phenotype is related to raised TMA levels – trimethylamine – due to mutations interrupting the FMO3 gene coding for flavin-containing mono-oxygenase 3. Mutant FMO3 transcript is present at a very low level as seen by RT-PCR analysis and this reflects active NMD. This gene defect is common in a breed of cattle and the loss of this gene also leads to fish odor syndrome in humans [134].

A matter of aroma: Interestingly, MOXD2 monooxygenase is a conserved mammalian factor expressed in olfactory epithelium – a nonsense mutation in exon 6 is partly responsible, amongst other alterations, for the loss of this factor [135]. Mutations of prokineticin 2 peptide (PROK2) and its seven-transmembrane domain type 2 receptor (PROKR2) are seen with these genes being involved in genesis of the olfactory bulb. Persons with one mutant allele may present with anosmia/hyposmia – two affected alleles present with a more extensive symptom complex [136]. Nonsense mutations are found in patients producing loss of PROK2 and PROKR2 factors.

Deficiency of sterol carrier protein X, SCPx, which is needed for breakdown of branched-chain fatty acids, leads to tremor, nystagmus and hyposmia. Leukencephalopathy is also present. A homozygous one nucleotide insertion leads to a frameshift and PTC [137].

A tactile matter - pain: Altered sensation due to genetic aberration such as nonsense mutation may reflect itself in altered perception to damaging external stimuli - especially to the skin - manifesting usually as a feeling of pain. An important part of the nervous system deals in terms of delivering physiological protective mechanism via the sensation of pain. Chronic pain states however rapidly may become debilitating and make for a significant burden.

Pain may be caused by inflammation (in arthritic conditions – which are chronic) or with ischaemia or by activation of superficial nociceptors through wounding so forth. Other conditions are represented by neuropathic pain (disease or damage to the nervous system proper) and psychogenic pain such as secondary to depression. Modulation of the pain experience is through memory or emotional link and therefore makes pain a highly complex neurological experience.

One particularly relatively common pain experience is that of migraine headache. The CACNA1a gene encodes calcium channel, voltage-dependent, alpha 1a subunit and is involved in a complex phenotype including migraine. Voltage-dependent calcium channels control entry of calcium ions into cells such as muscle and neurons. CACNA1a is mainly expressed in neuronal tissue. Mutations are involved in familial hemiplegic migraine and episodic ataxia 2. Interestingly, the gene carries the (CAG)n-repeat element. This encodes a polyglutamine tract that may be associated with disease. Expansion of the (CAG)n-repeats from usual 4-16 to 21-28 for the coding region is associated with spinocerebellar ataxia. Interestingly, Huntington's disease is part of a group of trinucleotide repeat disorders - (CAG)n. A sequence of 36 or more glutamines produces a Huntingtin protein with different characteristics and the altered form affects neurons. Larger repeats lead to more severe disease and very early age onset (<20 years of age). This is a neurodegenerative disorder and leads to cognitive decline and muscle coordination issues. Hence, CACNA1a represents a significant cornerstone for neurological homeostasis along with other repeat-related factors. A novel nonsense mutation in CACNA1a at codon 583 was identified in a patient presenting with attacks of both episodic ataxia and familial hemiplegic migraine. Acetazolamide (a carbonic anhydrase inhibitor and diuretic) alleviated the ataxia and migraine. Therefore this nonsense mutation nonsense is very clinically significant, and the pain attacks are debilitating in the absence of medical management [138]. Clearly, the truncation is removing essential portions of the factor or inviting NMD. Another earlier report is also consistent with nonsense interruption of CACNA1a leading to migraine and ataxia – weakness manifests during the attacks as a hemiplegia [139].

Pain may be manifest secondary to conditions such as severe osteoporosis. Osteoporosis-pseudoglioma syndrome is a rare disorder with early visual loss and juvenile osteoporosis. This is due to mutations in the low-density lipoprotein receptor-related protein 5 gene. A homozygous nonsense mutation (p.R1002x) was located in this gene in a patient with the syndrome. Bisphosphonate medical therapy improved bone mineral density and decreased the pain secondary to the low mineral quality of the bone. Readthrough, commenced early, may have rescued this patient's severe phenotype and prevented visual loss and bone-related pain [140].

On the other side of the coin, pain insensitivity is a serious condition. Being unaware of damaging stimuli may have drastic consequences as pain is often the mechanism used by the body to avoid noxious/dangerous external challenges. Congenital insensitivity to pain with anhidrosis (deficiency in perspiration) is a rare autosomal recessive complex with mental retardation. This is caused by mutations in the neurotrophic tyrosine kinase receptor type 1 gene and a combination of null, substitution nonsense mutation and frameshift, alleles leads to severe phenotype [141]. Nonsense mutations in another gene, SCN9a result in absence of pain. Sodium channel protein, SCN9a,

mediates voltage-dependent sodium ion permeability within excitable membranes and plays a role in pain, especially inflammatory related pain [142]. Interestingly, activating mutations produce severe episodic-related pain [142].

Amlexanox, the novel readthrough agent I propose in this present article, has the dual added property of inhibiting ikkepsilon and TBK1 kinases thus targeting diabetes and obesity [1]. It has been shown that inhibition of ikk activity (as ikk – IkappaB kinase – phosphorylates IkappaB and in leads in turn to NF-kappaB activation with nuclear translocation) prevents injury/infection induced upregulation of proinflammatory genes and thus may reduce hyperalgesia with inflammation [143]. Ikk inhibition prevented nuclear translocation of NF-kappaB and resultant NF-kappaB responsive gene upregulation such as COX2 and TNFalpha. Thus Ikk is a target for inflammatory related pain medications.

In the NF-kappaB pathway, p50 is an integral component with the RelA factor as a heterodimer that on activation is released from inhibitory IkappaBalpha. This is part of the standard or canonical signaling pathway. Synthesis of factors of the non-canonical pathway of activation/control of NF-kappaB, viz: RelB and p52 are in fact controlled by the canonical axis and are to some degree thus interlinked [144]. Tactile-related pain/nociception is absent in diabetic NF-kappaB double p50 knockout mice which suggests an important role for NF-kappaB in diabetic neuropathy states. Sulfasalazine treatment of diabetic animals reduced NF-kappaB p50 expression in sciatic nerves and dorsal root ganglia [145]. Diabetic neuropathy is represented, in part, by mechanical hyperalgesia and 'tactile allodynia', pain in response to non-painful stimulation. Clearly, this is a condition that requires medical attention in its own right to improve quality of life. Amlexanox, by targeting the non-classical pathway of NF-kappaB activation may well be of use for management of this condition in addition to sulfasalazine.

Consistent with this is that inhibition of ikkepsilon kinase may be used to treat inflammatory hyperalgesia [146]. Ikkepsilon has a high degree of homology to canonical IkappaB kinase subunits: Ikkalpha and beta. Ikkepsilon is highly expressed in nociceptive neurons as shown through mice studies in spinal cord and dorsal root ganglia. Ikkepsilon contributes to inflammatory-mediated hyperalgesia and not for other nociceptive stimuli. Ikkepsilon deficient mice had reduced inflammatory-related activation of NF-kappaB and reduced COX2 and inducible NO synthase. Therefore, ikkepsilon is a target for management of inflammatory-related pain/nociception and indeed as Amlexanox has the property of ikkepsilon inhibition [147] then it ought to be considered and trialed for use against inflammatory nociception.

Overall, readthrough and NMD with Amlexanox may have considerable benefit for various pain conditions and altered pain perception states. Modulating NF-kappaB activity would place Amlexanox in a strategic position in respect to homeostatic control of this central neurophysiological control axis. This could well have added 'side' benefits for management of inflammatory-related pain states and diabetic-related pain conditions.

This section indicates the uses for Amlexanox in managing conditions - including pain – associated with the five primary senses. These proposals for Amlexanox use are novel and significantly add to the 'target repertoire' of this drug (Figure 6).

Communicable disease and nonsense mutation

An introduction to this topic has already been provided [1] and I seek here to extend this discussion.

Viral infection

Measles: The matrix M protein gene of the measles virus was found to carry a PTC - truncating mutation, in a patient with subacute sclerosing panencephalitis (SSPE) [148]. It may be considered that this mutation led, at least in part, to the development of encephalopathy. Other analyses have shown that defective M matrix protein is associated with SSPE [149]. M proteins of Niigata-1 and ZH measles strains possessed nonsense mutations at nucleotide positions 68 and 96, respectively. Altered levels of M protein therefore may be said to be related to SSPE virulence.

Influenza: Mx proteins are antiviral and are induced via interferons in many mammals. These factors are part of the dynamin-like large GTPases involved in intracellular vesicle trafficking/organelle homeostasis. Such factors are active against RNA viruses like the influenza viruses. The mouse Mx1 GTPase resides in the nucleus and inhibits influenza virus replication. Human MxA GTPase is located in the cytoplasm and with bunyaviruses MxA prevents replication and with Thogoto virus MxA prevents nuclear viral transport. Generally, Mx GTPases sense nucleocapsid structures and redirect viral particles to compartments where they cannot effectively replicate [150]. Human cytoplasmic MxA protein results in anti-viral effects to influenza virus and thus inhibits cytoplasmic viral replication [151].

Mouse strains with mutated Mx1 gene demonstrated a higher susceptibility to influenza attack [152]. The Mx- phenotype of strain CBA/J is due to a nonsense mutation, viz: p.Lys389x. Mx mRNA levels in interferon-stimulated CBA/J cells are deregulated ~15-fold as oppose to interferon-treated wild-type (Mx+) cells. This can be taken to indicate that NMD is active when PTC mutation exists within Mx1. Clearly, this strain of mouse would be ideal to test the ability of readthrough with an agent such as Amlexanox, which also inhibits NMD [1]. Overall, this would serve as an excellent experimental model to examine readthrough as a viable option for prevention of nonsense-mediated human viral diseases.

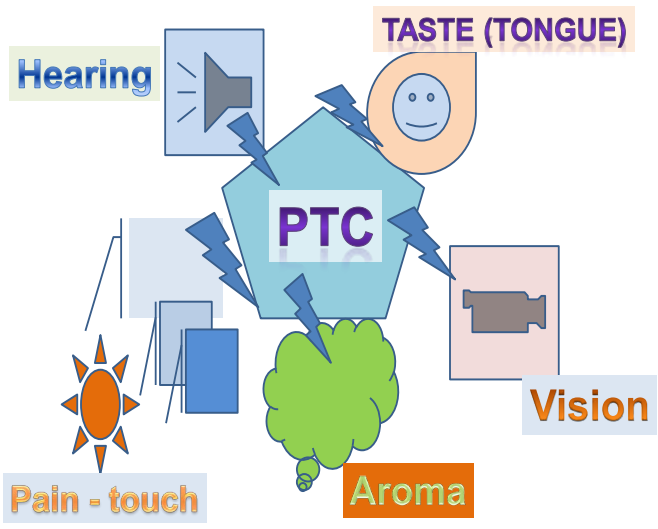

Figure 6: Nonsense mutations may relate to alteration in the five senses.

Herpes virus: TRIF factor (TIR-domain-containing adapter-inducing interferon-beta) is a downstream adapter in the Toll-like receptor (TLR) activation cascade involved with innate immunity. TLRs form response elements to pathogen invasion (see Section: Amlexanox and innate immunity). The result of activation of TLRs is the production of reactive inflammatory cytokines and chemokines. TLRs possess a TIR-domain that interacts with TRIF adapter and commences the signaling cascade. The transcriptional factor: NF-kappaB is a downstream activated complex from TRIF mobilization. As TRIF is required for raising response to infectious agents it is not surprising that nonsense mutations in TRIF gene are detrimental [153]. Herpes viruses, commonly blamed for the typical 'cold sore' may have more serious consequences for patients. Herpes Simplex Encephalitis (HSE) is the most frequent sporadically presenting type of childhood viral encephalitis. TRIF deficiency may predispose to paediatric HSE. In an autosomal recessive disease presentation a homozygous nonsense mutation produced a complete absence of TRIF factor. In this instance, both TLR3 and the TRIF-dependent TLR4 signaling pathways were ineffective in prompting downstream signaling cascades. Interestingly, TRIF-deficient patients with HSE upon follow-up suffered from nil other infection [153]. It ought to be noted though that clinical penetrance was found to be incomplete – certain HSV-1-infected TRIF-deficient family members did not develop HSE. It would appear then that TRIF is important in TLR3-dependent manufacture of anti-viral interferons within the central nervous system in primary HSV-1 paediatric infection.

Beyond TRIF, loss-of-function mutations in TLR3 itself and TRAF3 and TBK1 and UNC93B1 have been associated with HSE [154]. UNC93B1 is a protein involved in innate immune response and is likely involved in TLR trafficking. TLR3 and 9 bind UNC93B. TRAF3 (TNF receptor-associated factor 3) is part of a family of proteins that associate with and transducer signals from TNF receptor superfamily. Specifically, it mediates signaling from CD40, a TNF receptor family member needed for activation of immune response. TBK1 (TANK-binding kinase 1) is a serine/threonine-protein kinase. TBK1 phosphorylates IkappaB complex which then marks this inhibitory unit complex for proteasomal destruction via ubiquitination pathway. This inhibitor normally stabilizes NF-kappaB in the cytoplasm in an inactive state and, once freed, may proceed to the nucleus and act as a transcriptional factor activating many prosurvival/inflammatory-related genes. It is as such denoted as a 'noncanonical' kinase for IkappaB – non standard pathway for activation, or accessory activation pathway (see Section: Amlexanox and innate immunity). TANK protein itself regulates NF-kappaB activation via interaction with TRAF. By binding to TANK, TRAF factors may be released to enable NF-kappaB activation to proceed. Therefore predisposition to HSE is due, in part, to dysregulation of the TLR3-typeI interferon pathway which is needed to develop immunity to HSV-1. In a genome-wide screen for mutants leading to HSV-1 susceptibility with an *in vivo* model – a causative nonsense mutation leading to penetrant susceptibility was found in Receptor-type tyrosine-protein phosphatase C gene (L3x) – encoding tyrosine phosphatase, CD45. In these animal models, expression of cytokines and inflammatory-related factors such as metalloproteinases 3 and 8 were highly increased in CNS. This led to out of control inflammation and secondary viral pathological damage. Thymocyte and B cell maturation were severely impaired. T cell transfer repaired immunity to HSV-1. Thus CD45 mediated T cell functional cross - talk is a central feature in the pathology of HSE. It would be most interesting to investigate readthrough for *in vivo* correction/rescue of the CD45 nonsense mutation in this animal model – again, this would provide a further proof of principle for the use of readthrough in management of human nonsense mediated communicable disease treatments.

Cytomegalovirus: Autosomal recessive hyper-IgE syndrome is a combined immunodeficiency showing a susceptibility to viral infections with atopic eczema and displays high serum IgE and defective T cell activation. Many cases are due to null mutations – amongst which nonsense mutations significantly figure in such genes as DOCK8 and TYK2 [155]. Bacterial, fungal and viral infections are the most frequent clinical features. CMV infection too was significant in correlating with poor prognosis – viz: early death. DOCK8 is a large protein involved in intracellular signaling (~190 Kda). It is a member of guanine nucleotide exchange factors – which function as activators of small G proteins. A patient diagnosed with autosomal recessive hyper-IgE syndrome was managed by haematopoietic stem cell grafting. She was found to carry a homozygous nonsense mutation in the DOCK8 gene [156]. The authors point out that early curative therapies would be most welcome to prevent serious complications of later infections – it would be interesting to trial readthrough therapy for patients such as this for nonsense-mediated Hyper-IgE syndrome. Bone marrow stem cell transplantation carries risks such as Graft vs Host reaction as well as veno-occlusive disease amongst other noteworthy issues.

HPV: HPV - human papilloma virus – is a DNA virus of the papillomavirus family and is productively infective only in keratinocytes of the skin/mucosa. Various types such as HPV16 and HPV18 can be oncogenic and HPV vaccines may prevent oncogenic-type HPV infection. Overall, HPV represents a significant public health problem.

The RHOH gene – Ras homolog gene family member H [157] – a member of Ras superfamily of small GTPases - is a small G-like protein only transcribed in haemopoietic cells. This factor is a regulator of thymocyte development and T-cell antigen receptor signaling and binds GTP but lacks GTPase activity. It also inhibits activation of NF-kappaB by TNF and IkkB. A deficiency of RHOH results in T cell dysfunction and susceptibility to HPVs causing the devastatingly disfiguring skin condition: epidermodysplasia verruciformis (EV). Further, an increased risk for skin cancer exists with such affected individuals. In a previous study, two related individuals were found to be T cell deficient with persistent EV-HPV causing infections – they both had homozygous mutation causing a stop nonsense mutation in RHOH gene. Expression of normal RHOH allele in Rhoh -/- haematopoietic stem cells rescued the T cell deficiency in mice and this suggests that T cells play a role in EV-HPV pathogenesis and also that the symptom complex is able to be rescued [157]. This also is very suggestive that readthrough again could, in such nonsense-mediated cases of HPV infection leading to EV, actually be used to effectively treat the condition.

A homozygous nonsense mutation, p.R115x, in the MST1 gene, encoding macrophage stimulating protein [158] produced a case with EV-HPV along with bacterial and fungal infections. T cell depression was noted.

Human papillomavirus represents a significant cause of gynaecological carcinomas [159]. Viral proteins E6 and E7 are regarded as oncogenic factors. They achieve this status via targeting important TS such as p53 and Rb (retinoblastoma) proteins. A particular isoform of the mixed lineage leukemia 5 gene (MLL5beta) regulates and activates the transcription of oncogenes E6 and E7 of HPV in infected epithelium. MLL5β downregulation restored p53

protein and reduced Rb phosphorylation. This then, in turn, activated apoptosis. The novel MLL5 isoform: MLL5β, is a result of an added 26 nucleotides that introduces a stop codon in exon 14 of MLL5. This then represents the truncated MLL5β isoform that associates with AP-1–binding site located upstream of the E6 and E7 promoter site. This association thence creates a complex that transcriptionally activates E6/E7. Authors suggest that MLL5β may be a useful therapeutic target - perhaps more so than E6 or E7 individually. Again, readthrough in this context may well aid prevention of the formation of this deleterious truncated isoform that appears to have gained oncogenic functional status.

As I have previously outlined for NOTCH1 [1], truncation may lead to significant gain of function that, in turn, could well be amenable to readthrough. In the context of gynaecological cancers, this concept too is relevant it would appear from the MLL5 analysis with HPV.

Infectious Mononucleosis: X-linked lymphoproliferative disease (XLP) is a genetic disease resulting in severe immunodeficiency. Epstein Barr Virus infection and malignant lymphoma may be hallmarks for this condition. It has been linked to SH2D1A gene (SH2 domain-containing signal transduction adaptor protein) mutations. This gene regulates T and B cell homeostasis and immune responsiveness. A young patient was diagnosed with XLP and his close relatives succumbed to fulminant EBV infection. A nonsense mutation, viz: p.Arg55x, was found in the SH2D1A gene [160]. Again, readthrough could have considerable value here in protecting against EBV infection in this context.

Immune-deficiency virus (IDV): Resting CD4+ T cells are resistant to IDV infection as oppose to productive, activated CD4+ T cells [161]. Reverse transcription of viral genomes is limited early on with the infection phase. Deoxynucleoside triphosphate triphosphohydrolase (SAMHD1) blocks reverse transcription of IDV RNA in resting T cells. Viral Vpx mediated proteasomal degradation of SAMHD1 allows productive infection. In an individual with Aicardi-Goutières syndrome homozygous for a nonsense mutation in SAMHD1, IDV infection was permitted. CD14+ cells express innate immune system factor, CD14. It acts as a co-receptor for TLR4 to detect bacterial LPS antigen amongst other pathogen-associated patterns. Cells expressing CD14 are macrophages, dendritic cells and monocytes. SAMHD1-deficient $CD14^{+}$ cells from Aicardi-Goutières syndrome sufferers are very susceptible to IDV infection [162]. The nonsense mutation p.R164x in homozygotes results in a lack of SAMHD1 and permits IDV infection. CD14+ immune cells were susceptible for IDV in this context. SAMHD1 therefore is presumed to be a potent lentiviral restriction factor of the host and any mutations, particularly those removing function entirely such as nonsense-type, are highly deleterious for the host. Clearly readthrough has a potential in this situation to rescue function and restore host IDV resistance.

Aicardi-Goutières syndrome is a congenital immune-mediated neurodevelopmental condition which may be accounted for, in part, by SAMHD1 mutations. The neurological deterioration generally is severe leading to cerebral atrophy. No clearly defined treatment is available. Perhaps readthrough in nonsense-mediated Aicardi-Goutieres syndrome may be of use and be preventive of productive IDV infection in these cases.

Readthrough of nonsense codons has to be carefully tailored. This is especially true for IDV. In viral replication, IDV reverse transcriptase is error-prone. Host APOBEC-driven hypermutation of viral Gag and Pol genes produces replication-deficient variants with truncating nonsense mutations [163]. Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G, APOBEC3G, is a member of the APOBEC protein family and plays a role in innate antiviral immunity. It is a cytidine deaminase enzyme and as such may create stop mutations in selected instances [1]. Thus, APOBEC produces anti-retroviral immune activity - however, viral infectivity factor (Vif) protein from IDV counteracts this by binding to APOBEC and enabling ubiquitination and proteasomal degradation. Paradoxically, APOBEC by contributing to IDV mutagenesis creates greater genetic diversity and supports IDV ability to adapt, perhaps, for example, in developing drug resistance. Nonetheless, having said this, replication deficient variants of IDV may be thus produced via the APOBEC surveillance system and ideally, selecting for these could enhance IDV clearance – readthrough may oppose this strategy by reducing replication-deficient IDV variants by nonsense rescue. A further consideration for readthrough has been outlined through activation of dormant endogenous retrocyclin activity [1]. Ultimately, the strategy of readthrough may need to be carefully individually focused in terms of IDV and whether retrocyclin activation will be over-riding in preventing IDV infection shall need to be assessed in model systems and clinical trials. As IDV has evolved in absence of retrocyclin selection then it is hopeful that readthrough may have a general benefit for IDV management.

Bacterial infection:

Anthrax: ANTXR1 (anthrax toxin receptor 1) is a transmembrane protein and a docking protein/receptor for Bacillus anthracis toxin which causes anthrax. Interestingly, mutations in ANTXR1 result in GAPO syndrome – Growth retardation Alopecia Pseudoanodontia Optic atrophy [164]. Homozygous nonsense mutations in ANTXR1 were found in persons with GAPO viz: p.Arg88x; p.Arg169x. Human ANTXR1 deficiency phenotype mimics that found in ANTXR1 mutant mice and the notion is that ANTXR1 is involved in extracellular matrix homeostasis. The mouse model could be engineered to mimic nonsense-mediated human GAPO and tested for readthrough in this context.

Rescuing GAPO syndrome by restoring ANTXR1 may make for anthrax susceptibility though as this receptor is a pathogenic element in anthrax invasion [165]. This matter would appear less of a concern than GAPO for those persons afflicted with that syndrome.

Bacterial adaptation: Importantly, it must be remembered, as a rule, that nonsense mutations may in fact confer a certain selective advantage for bacterial adaptation to new environments [166]. Loss of function or null mutations which include nonsense point substitution mutations, aid adaptation to novel environments via a regulatory/metabolic rewiring mechanism. Such adaptive null mutations are being more greatly appreciated in natural as well as in clinical settings. A prime example is null mutation mediated adaptation which aided the divergence of B. anthracis from a B. cereus ancestor. B. anthracis carries, in addition to two virulence plasmids, an ubiquitous nonsense mutation in plcR which produces a pleiotropic transcriptional regulator. The plcR null mutation leads to reduction in degradative enzymes and virulence factors. The notion is that plcR inactivation is part of a greater co-evolution of the genome and the two virulence factor plasmids that ultimately delineated B. anthracis as a separate species. Many other cases exist in this type of null mutation release from potential mal-adaptation to novel environments.

Other examples were presented of nonsense mutations in

bacteria leading to increased virulence directly [1]. The subtlety with adaptation is that nonsense mutations may, by modulating virulence factor expression, lead to adaptation of the bacterial spp, which in the ultimate, is likely favourable in terms of survival and ecological development. This would appear a common phenomenon for bacteria – again, readthrough may prevent this type of adaptation and ultimately prevent bacterial divergence into alternate species that may well prove to be more virulent or: 'environmentally-adapted'.

It may well prove to be the case that fungi and viruses, and other pathogens, may employ similar mechanisms that require further delineation and exploration and that may well form ripe targets for readthrough action.

Fungal infections: Drug resistance [167] is a growing problem with management for C. tropicalis, a significant Candida isolate. FCY2 encodes a purine-cytosine permease and loss of heterozygosity in FCY2 heterozgotes with a nonsense mutation led to anti-fungal drug resistance. Nonsense mutation in the ERG6 gene leads to increased resistance to polyenes in C. glabrata [168]. This isolate provides a good resource to test readthrough therapy for enhancing drug sensitivity within fungal isolates of clinical relevance. Further, in clinical isolates of C. lusitaniae FCY2 gene disruption via nonsense mutation led to flucytosine and flucytosine/fluconazole cross-resistance [169]. Clearly, this supports the use of readthrough for enhancing drug susceptibility in Candida spp. These investigations also a novel *ex vivo* testing ground for readthrough agents that may be later clinically directed.

Mucocutaneous fungal infections may be readily seen in persons with nil underlying immunological defects. In one particular family several individuals were found that suffered fungal infections. Mutation: p.Tyr238x, a nonsense mutation in the beta-glucan receptor: dectin-1, was isolated in these cases [170]. Mutated dectin-1 was not capable of beta-glucan binding and led to defective production of cytokines viz: Il-6/TNF/Il-17 on challenge with beta-glucan or C. albicans. Nonetheless, fungal phagocytosis was normal and this suggests that dectin-1 deficiency is not associated with invasive fungal attack, rather, this supports a role of dectin-1 in surface antifungal defence.

Genetic susceptibility to fungal infections has been noted and chronic mucocutaneous candidiasis may present with autosomal dominant or recessive patterns of inheritance [171]. Four affected members of a large consanguineous family presented with homozygous nonsense mutation, p.Q295x, in the CARD9 gene (Caspase recruitment domain-containing protein 9). The affected persons presented with low numbers of Th17 cells (helper T cells producing Il-17). This blocks signaling from the fungal pattern receptor dectin-1 and is therefore part of that cascade – again, consistent with the phenotypic presentation of the disease.

Dental infections: As a dental surgeon, I am particularly interested in the potential of readthrough, more specifically, use of Amlexanox, in management of dental infections.

Fungal infections are a constant consideration in oral pathology. As noted above, nonsense mutations may be associated with anti-fungal drug resistance. C. dubliniensis may be isolated from immunocompetent individuals and is closely related to the commonly found mouth fungus, C.albicans and is thus most frequently found in the mouth. Loss of function mutations leading to premature truncation of CdErg3p gene led, in a number of isolates, to diminished sterol C5,6-desaturase synthesis. This in turn led to itraconazole anti-fungal drug resistance [172].

Already, I have discussed nonsense mutations in Cathepsin C relating to Papillon–Lefèvre syndrome which presents with the rapid periodontal destruction in primary and secondary dentitions [1]. So clearly, readthrough has a significant potential place within the armamentarium of the dental profession for management of certain dental infections of relevance to the patients we see.

In this section I have highlighted the evidence suggesting that nonsense mutations determine many suited targets for readthrough therapeutics in communicable diseases. In this context, Amlexanox may offer a considerably new approach in terms of infectious diseases management (Figure 7).

Diabetes

Amlexanox is an agent that not only produces readthrough but also targets diabetes directly (v.i.). Here I present various aspects of diabetes in relation to nonsense mutations that in turn may form suited Personalised readthrough targets. I have already given some background to this topic [1].

Diabetes insipidus (DI): Although not related in causation to the more commonly described diabetes mellitus (DM), this condition merits outlining under this topic heading. In DI, there is a problem with production of antidiuretic hormone (central DI) or in turn, due to an issue with the kidney's response to this factor (nephrogenic DI). Blood sugar levels are not directly disturbed as in DM.

Antidiuretic hormone retains water in the body and constricts blood vessels. It acts to increase water absorption in the collecting ducts of the kidney nephron and is produced as a peptide hormone and stored in the posterior pituitary gland until released.

Alterations of the A VPR2 gene encoding the AVP V2 receptor results in congenital nephrogenic diabetes insipidus. Activation of the V2 receptor is via vasopressin (antidiuretic factor) which in turn causes trafficking of the water channel AQP2 to the luminal plasma membrane – a percent of this factor is duly excreted. A novel nonsense mutation, p.W296x of A VPR2, eliminated measurable excretion of AQP2 yet a missense mutation, p.V88M in other family members still allowed for

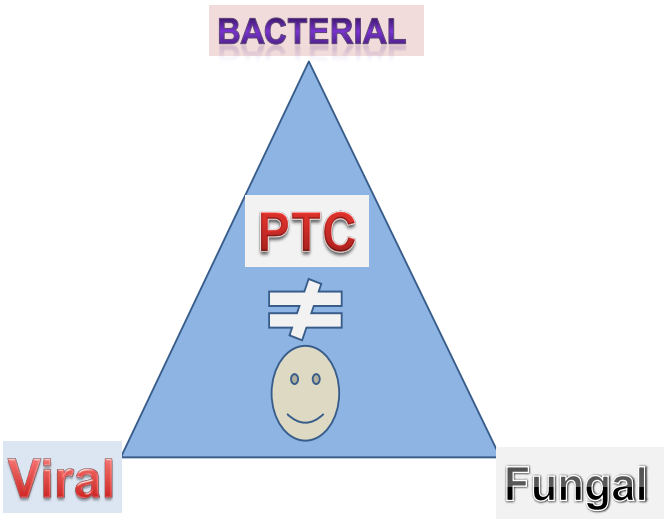

Figure 7: Nonsense mutations may lead to viral/bacterial/fungal infection/ pathologies.

AQP2 detection in the excretion. Nonsense mutation produced a more severe phenotype [173].

Central-type DI is an autosomal dominant inherited disease presenting with a deficiency in vasopressin. The gene for vasopressin has three exons and in one family with this disease, a novel heterozygous nonsense mutation in exon3, p.Gln83x, was found in all family members afflicted with DI - the mutation was not present in healthy family members [174].

Many human diseases are due to inactivating mutations in specific G-protein-coupled receptors - GPCR - and in ~10% of cases a PTC leads to a truncated inactive receptor. A particularly interesting study has shown proof of concept for readthrough for DI is definitely a reality [175]. A mouse model system carries the mutant V2 vasopressin receptor with inactivating p.E242x mutation - a situation closely mimicking human X-linked nephrogenic diabetes insipidus. Readthrough *in vivo* with aminoglycoside G418 increased water re-aborption and improved clinical outcome measures. This investigation is very strong evidence supporting use of readthrough for nonsense-mediated GPCR diseases, of which DI may represent an example.

Diabetes mellitus [DM] – early onset: Already, I have indicated that nonsense-mediated DM may form a suited target for readthrough [1]. Also, I have alluded previously to the notion that substitution nonsense mutations may be seen to be related to premature ageing phenomena such as neurological deterioration [1] and osteoporosis (see above), heart disease and indeed cancer itself [1]. Diabetes may be no exception to this relationship. A mouse model of early-onset renal failure shows that the causative mutation is a xanthine dehydrogenase nonsense mutation [176]. Renal problems are oftentimes a hallmark of diabetes generally and may act in concert to worsen the prognosis. The mouse model for premature renal deterioration is well suited to attempt readthrough rescue of same – so as to further demonstrate the benefits and practicality of this approach in the context of diabetes.

Wolcott-Rallison syndrome is an autosomal recessive condition which results in early onset diabetes and early osteoporotic fractures. Alterations of the eukaryotic translation initiation factor 2alpha kinase gene are causative and a case demonstrated a homozygous nonsense mutation (p.R491x) affecting that gene [177]. A novel nonsense mutation in glucokinase, pC364x, has also been found to relate to diabetes type II phenotype in younger age onset cases [178].

For these varying early onset examples of diabetes readthrough could certainly prove to be of significant value. Amlexanox therapy may offer up novel approaches to such clinical problems.

Amlexanox and innate immunity

Introduction: Amlexanox is an anti-inflammatory medication and as such has been used for over two-decades for managing mouth ulcers [1]. Inflammation, although a normal host response to infection and/or injury, may also be related to tissue damage and disease. It has been also implicated in the formation of obesity-related insulin resistance. The noncanonical IkappaB kinases, viz: ikkepsilon and TBK1 are elevated in obesity and insulin resistance – and are thus considered to be productively involved in the development of same. Amlexanox prevents an animal model from gaining weight whilst fed on a high fat diet. Further, Amlexanox administration to animals which were genetically obese returned the body weight to normal. This drug achieves increases in energy expenditure via increased body heat – viz: thermogenesis. Thus the hallmark of obesity and insulin resistant diabetes may be proposed to be dysregulation of inflammation which in turn dysregulates energy balance. The noncanonical NF-kappaB kinases: Ikkepsilon and TBK1 are expressed to sustain energy storage with insulin resistance. Amlexanox, through inhibition of these two kinases, reduces inflammation by perhaps elimination of the feedback loop that keeps inflammation at low but constant level.

The very targeted anti-inflammatory response with Amlexanox is certainly an advantage over simply disabling an entire arm/branch of the inflammation pathway as general anti-inflammatory medicines do [179]. Nevertheless, this modulation of inflammation must be balanced in terms of what effects that could have in terms of pathogen exposure, a subject I shall discuss.

In many ways, the dual nature of Amlexanox is interesting. Readthrough along with noncanonical kinase inhibition both may be seen as properties that may be used to advantage. This dual nature of Amlexanox appears thus far unique, insofar as there are medications that either perform readthrough or modify the inflammatory response, but not effectively both together. Harvesting this dual characteristic of Amlexanox could well prove to be highly strategic for general medical use, despite the fact that in the long term, response to viral or other pathogens shall have to be carefully assessed. The sections below outline in further detail these characteristics and associated caveats in terms of use.

Amlexanox and inflammation and NF-kappaB: An inflammatory link exists between obesity and insulin resistance [180]. Obesity produces a state of chronic, low-grade inflammation in both the liver and adipose tissue which is accompanied by the local secretion of cytokines and chemokines that block insulin action. By the blockage of inflammatory pathways one may disrupt obesity buildup and insulin resistance. Clearly, there is a tie between inflammation and type II diabetes with its attendant obesity-related phenotype.

The transcriptional factor NF-kappaB is found to be activated adipose tissues and as such is likely to be involved in development of insulin resistance. Noncanonical kinases TBK1 and Ikkepsilon are regulators of NF-kappaB and are found to be increased in adipose tissues in animal models during a high-fat diet and relate to insulin resistance [180]. Amlexanox inhibits these noncanonical kinases but has no effect on IKK-α or IKK-β (canonical NF-kappaB kinases). It ought to be noted that TBK1 and Ikkepsilon share an overall 65% sequence similarity and bear 72% similarity in the ATP-binding region. By competition studies, Amlexanox has been found to interact with these kinases at the ATP-binding site. This drug was found to increase the phosphorylation of TBK1 at Ser-172 in adipocytes and blocked polyI:C-stimulated phosphorylation of interferon responsive factor-3 (IRF3), a likely substrate of Ikkepsilon and TBK1 and antiviral factor [180].

Inhibition of Ikkepsilon and TBK1 by Amlexanox does not appear to be involved in directly blocking the inflammatory effects of cytokines/LPS on inflammatory signaling or the NF-kappaB pathway. Rather, feedback inhibition is prevented. This feedback is normally provided by TBK1 and Ikkepsilon. It would appear that the noncanonical kinases are not strictly performing as standard, canonical, IκB kinases. In other words, they are not directly inflammatory-related. In fact, noncanonical kinases may seek to dampen the inflammatory response. These kinases therefore may be involved in generating low-grade inflammation that does not resolve and may lead to type II diabetes with obesity [180]. By targeting these kinases, resolution of the inflammation may occur, which is beneficial.

An immediate concern though with the promotion of long-term Amlexanox use, is that of disturbing host immune system. The noncanonical kinases, as noted above, have antiviral factors such as IRF3 as substrates. The relevance of such kinases and their substrates to pathogens that may potentially invade a host ought to be explored to determine the relevance of this pathway to essential host defence.

Pathogen exposure and noncanonical NF-kappaB kinases: Pathogens have evolved to divert the innate immune system response such as to dysregulate and lessen this intrinsic defence mechanism. The innate immune system involves TLR – Toll-like receptors coupled with an elaborate signaling network involving NF-kappaB activation. Any factor that modulates or interferes with this system such as kinase inhibitors like Amlexanox impacting on NF-kappaB may be said to be disturbing the response of the innate immune system.

Viruses and noncanonical NF-kappaB kinases: Poxvirus [181] have evolved a clever way to help evade host innate immunity. Toll-like receptors are intimately part of the signaling cascade of the innate immune pathway. These serve as pathogen pattern recognition receptors and partner with IL-1 domains to initiate the flow of events culminating in NF-kappaB and IRF3 activation. This then also serves to initiate proinflammatory cytokine production. Vaccinia virus produces N1L, a factor involved in disrupting the chain of events leading to signaling secondary to TLR/IL-1 interaction. Poxvirus contains a highly homologous factor to N1L. This factor is notable in that it strongly interacts with TBK1. As such, it targets the IKK complex for inhibition. N1L inhibits IRF3 signaling, also regulated by TBK1. Thus, N1L is a virus-designed immune-modulator of innate immunity and disrupts antiviral mechanisms via NF-kappaB and IRF3.

IDV also has its own mechanisms for dealing with anti-viral host innate immune responses. IDV infection of macrophages and T cells fails to alert the innate immune response with type I interferon production. Cytosolic exonuclease TREX1 reduces interferon produced on response to IDV. TREX1 interacts with cytosolic IDV DNA not imported to the nucleus and removes any excess IDV DNA that could initiate interferon expression in a pathway that depends on TBK1 and IRF3. The virus therefore hijacks host factors to its own end to evade the innate immune system [182].

TLR-ligand activated cells produce anti-viral factors that arrest IDV upon macrophage infection but this mechanism does not come into play for T cells infected by IDV. TBK1 is needed in macrophages to result in an antiviral response. This is thus cell-specific and requires TLR-linked antiviral activity via intermediates such as TBK1 [183]. TBK1 also decreases IDV viral budding and spread – independent of interferon [184].

In innate immunity, initial detection of invading foreign or viral nucleic acid is needed to mount the inflammatory response that is productively antiviral. A DNA-protein kinase forms a DNA sensor that activates innate immunity. DNA-PK acts as a pattern recognition receptor and binds cytoplasmic DNA and initiates production of interferon type I and cytokine and chemokines dependent on IRF3 and TBK1 and stimulator of interferon genes (STING). DNA-PK is DNA virus-type specific and does not respond to RNA virus particles. Further, DNA-PK is involved in DNA repair and V(D)J recombination and its absence marks severe combined immunodeficiency (SCID) [185].

IDV Vpr protein is important in viral replication. Vpr moderates the NF-kappaB pathway. IDV infection increases IkappaB alpha phosphorylation induced via Vpr and Vpr is capable of activating both canonical and noncanonical pathways. Vpr is present in IDV particles and so activates NF-kappaB on immediate entry to the cell [186].

The IDV trans-activating Tat protein is needed for viral replication and induces cytokines from the host such as IL-10. IL-10 is anti-inflammatory and produced in IDV infection whereby IDV induces an immune-modulation of its own. Tat induces IL-10 in monocytes via NF-kappaB pathway. Noncanonical and canonical pathways are involved [187].

The innate antiviral immune response is rapid and mediated via type I interferons that are restrictive to viral replication. After viral elimination the system is 'turned off' – a self-regulating or homeostatic mechanism by the host. RING-domain containing protein 11 is a negative regulator of innate immunity – this ring protein binds to TBK1/Ikkepsilon and blocks their ubiquitination. RING protein interacts with an adapter to achieve this and thus blocks antiviral signaling and interferon-beta production [188]. Hence, ubiquitin plays a role in both NF-kappaB activation as well as termination of antiviral signaling. Removal of ring protein 11 by knockdown enhances interferon-beta promoter activation by the noncanonical NF-kappaB kinases. So Ring factor 11 would appear to target these kinases for inhibition. Ring protein 11 by inhibiting noncanonical kinase activation dampens antiviral signaling and interferon-beta production as a means for homeostatic control. Polyubiquitination of noncanonical kinases activates them for antiviral mechanisms – ironically, this does not target them for destruction such as with NF-kappaB inhibitor removal.

Optineurin is a protein that may play a role in glaucoma formation and this factor appears to negatively regulate the antiviral response. Optineurin forms a complex with TBK1 and the ubiquitin ligase, TRAF3 [189]. Thus it could well form a target for antiviral therapies to release TBK1 from inhibition. Again, this seems to act as a host self-regulating mechanism to prevent excessive and unwanted tissue inflammatory damage on viral exposure.

Hepatitis B virus (HBV) is known as a stealth virus – it invades and replicates in the liver unseen by host innate immune systems. TypeI interferon is evaded by HBV replication in hepatic cells – due in part to HBVx protein impairing the interferon-beta promoter and viral sensors. HBx is a deubiquitinating enzyme and it deconjugates TRAF3 releasing it from TBK1. HBx also directly interacts with TBK1, ikkepsilon and IRF3 [190].

Rabies virus phosphoprotein is a typeI interferon antagonist. It achieves this by blocking use of IRF3/7 as noncanonical kinase substrates. Viral mutants lacking key protein domains were non-pathogenic. Neuro-virulence of interferon-resistant rabies virus thus may relate to the capacity of the virus to prevent IRF3/7 activation [191].

HSV may be life threatening in immune-compromised patients. HSV1 induces a persistent activation of NF-kappaB in early phase infection. Active NF-kappaB in turn enhances HSV1 gene expression and this mechanism is not blocked by the antiviral agent acyclovir. Inhibiting canonical kinases by the anti-inflammatory natural derivative: cyclopentenone prostaglandin A(1) blocks HSV1 gene expression and reduces replicative viral load over three-orders of magnitude. Cyclopentenone prostaglandins inhibit canonical ikkbeta kinase and thus tunes down NF-kappaB. Ikk kinases therefore may form a target for anti-herpetic drugs. Homology between canonical and noncanonical kinases suggests that there is a relation here that may

be exploited in moderating HSV infection via targeting either of these pathways by selective drugs [192].

Overall, in terms of virus infection, the effects of the noncanonical kinases are complex. They form a basis for antiviral defence that may be subverted by viral machinery and further, these kinases may form a self-regulatory mechanism to provide protection from excessive host antiviral response. The nett effect of modulating noncanonical kinase cascade long-term with such agents as Amlexanox shall need to be obviously assessed carefully via clinical trials. These trials shall need to be carried out under a variety of circumstances such as with individuals who have altered immune status and those who are virally infected.

Bacteria and noncanonical NF-kappaB kinases: In macrophages, noncanonical kinases operate the IRF3/7 transcriptional factor response [193]. Interferon is a central effector of the innate immune response. It is induced via TLR and non-TLR means from bacterial and viral antigen presentation. LPS from bacteria induces TBK1 and Ikkepsilon to result in interferon production. TBK1 kinase is induced rapidly after LPS stimulation with Ikkepsilon following on later. Targeting noncanonical ikks via RNA interference in macrophages prevents interferon-beta and IRF7 expression after bacterial LPS stimulation.

LPS from Salmonella spp and Haemophilus influenzae induced interferon-gamma release via TLR4/TRIF/Ikkepsilon-TBK1 circuits in T-helper cells of nonsmokers. This effect involving noncanonical kinases was however somewhat blunted in smokers which may account for more productive infections in this subset of individuals [194].

Nucleotide binding oligomerization domain 1 (NOD1) plays a role in host defence at mucosal surfaces. A ligand specific for NOD, which is a peptide from bacterial peptidoglycan, results in production of typeI interferon. Participating in this signal circuit is TBK1 and Ikkepsilon which lead to activation of IRF7. This in turn leads to interferon-beta production and thence downstream chemokine expression. Interference with this pathway leads to Helicobacter pylori susceptibility. Chemokine production results in chemoattraction for monocytes, macrophages, T cells, NK cells and dendritic cells which all provide for defensive aid [195].

An intriguing parallel may be made between BX795 and Amlexanox. BX795 represents an aminopyrimidine compound (N-(3-((5-lodo-4-((3-(2 thienylcarbonyl)amino)propyl)amino)-2 pyrimidinyl)amino)phenyl)-1 pyrrolidinecarboxamide. This is a potent ATP competitor and reversible kinase inhibitor. Notably, this compound inhibits phosphorylation at Ser-396 and nuclear translocation and transcriptional activity of IRF3. BX795 has no effect on canonical NF-kappaB signaling axis. This drug though is a specific inhibitor for the noncanonical kinases, TBK1 and Ikkepsilon. As such, it is involved in blocking of phosphorylation and nuclear migration along with activity of IRF3. Therefore, it interferes with interferon-beta production in macrophages on appropriate stimulation, such as by bacterial LPS. BX795 blocks phosphorylative autoactivation of stimulated noncanonical kinases at Ser-172. It does not block phosphorylation of basal level noncanonical kinases however, in response to stimuli such as LPS. Paradoxically though, BX795 actually increased the level of phosphorylation at Ser-172 in response to stimuli. This suggests that, *in vivo*, kinase(s) upstream to the noncanonical kinases are capable of productively activating these. Another key aspect that must be understood is that the noncanonical kinases are able to negatively feedback control their own activation to limit over activation secondary to stimuli such as LPS [196].

As mentioned above (Amlexanox and inflammation and NF-kappaB) Amlexanox inhibits noncanonical kinases but does not appear to target the canonical NF-kappaB kinases. There is a relatively high degree of homology between the two noncanonical kinases, viz: TBK1 and Ikkepsilon – particularly in respect to the ATP-binding region. Amlexanox interacts at this site. Indeed, this drug increases phosphorylation of TBK1 at Ser-172 and blocks phosphorylation of IRF3 [180]. As also stated above, inhibition of Ikkepsilon and TBK1 by Amlexanox does not block proinflammatory effects of cytokines/LPS. Feedback inhibition is prevented - a mechanism normally provided by TBK1 and Ikkepsilon – which probably, also as indicated above, is a means to self-regulate and prevent over activation of these kinases and the inflammatory axis they control.

BX795 is a small cell-permeable kinase inhibitor – like Amlexanox. Notably, noncanonical kinases are needed for activation of transcriptional factor IRF3 and production of type I interferons. These kinases thus activate NF-kappaB dependent transcription of a brand or section of the proinflammatory gene axis. BX795 reduced IRF3 activation and interferon-beta production but had nil effects on NF-kappaB activation by various stimuli. This is consistent with mouse knock-out studies that show that absence of noncanonical kinases had no effects on NF-kappaB activation in reaction to various stimuli – such as bacterial LPS. There appears to be perhaps a redundancy in this respect with the canonical kinases. Nevertheless, this does not mean that the noncanonical kinase signaling platform is not required – it appears relevant to production of anti-viral factors certainly. The significance of inhibiting these kinases is highly relevant in terms of proposing long-term Amlexanox use for either nonsense readthrough or obesity/insulin resistance control. Ultimately, long duration clinical trials are going to provide the definitive answer to this central point.

Overall, it may be argued that there rests a degree of complexity of involvement with the noncanonical signaling axis in innate immunity. Clearly, this axis plays a central role in viral defence – in bacterial defence too there is a role being played here. Doubtlessly, further careful trials with other drugs targeting this axis are really needed to definitely probe the action of the non-standard NF-kappaB kinase activation platform on bacterial and, indeed, other pathogen challenge.

Periodontal disease, Ikkepsilon, TBK1 and heart disease: As a dental surgeon, I am interested in the progression and pathology of periodontal disease – a bacterially-related process that results in destruction of tooth-support. My aim is to diagnose and advise on management strategies for this disease with the patients I see. There would appear to be, from the above discussion, a certain degree of relevance that may be attached to noncanonical IkappaB kinases and TLR to periodontal infection. I also consider the importance inflammation, specifically that mediated through the noncanonical kinase circuit to cardiac and vascular disease. It has been inferred in the literature that periodontal disease may relate to cardiac and vascular disease risk [197]. In this respect, the likely tie between periodontal-induced inflammation and vascular disease is worthy of presentation, particularly in the context of the noncanonical NF-kappaB axis – where novel repurposed agents such as Amlexanox may well prove to offer valuable contributions to the armamentarium of drugs aimed at disconnecting paths involved in damaging chronic inflammation.

Periodontal disease and TBK1: Surface molecules of pathogens form 'patterning elements' which stimulate, in turn, host immune responses. MspTL is the abundant outer membrane protein present on T. lecithinolyticum – a pathogen associated with periodontal

disease. This protein induces various host proinflammatory cytokines. Further, bacterial LPS and flagellin also induce interferon-beta. MspTL treatment of a human monocyte cell line and primary cultures of gingival fibroblasts induced interferon-beta and interferon gamma inducible protein 10 (CXCL10 – a chemokine cytokine - chemoattractant for macrophages, T cells, NK cells and dendritic cells). MspTL activated TBK1 but not Ikkepsilon and also resulted in dimerization of IRF3 with nuclear translocation. Thus, this outer membrane protein was capable of inducing interferon-beta and CXCL10 along with RANTES (a chemokine that attracts T cells, basophils and eosinophils) by means of the TBK1 noncanonical kinase signaling axis [198]. It ought to be noted that chemokines, such as CXCL10 may pressage a harmful level of immune activation. In IDV, CXCL10 correlates positively with rapid decline [199] and correlates with poor outcome for Hepatitis C virus infection [200]. Thus, inflammatory pathway activation via TBK1 may be deleterious and inhibition of this signal platform on pathogen exposure may prove to be beneficial, not just in terms of moderating local periodontal destruction but also for controlling various systemic reactions. TBK1 targeting agents such as Amlexanox may well prove beneficial when seen in this context.

Periodontal disease and Ikkepsilon

Daidzein is an isoflavone found in plants, herbs, soybeans and soy products such as tofu. Daidzein inhibits LPS of the periodontopathic bacterium P. intermedia from inducing interleukin-6 and other proinflammatory messengers from LPS treated host derived mouse macrophages. This natural compound also prevented degradation of IkappaBalpha induced by P. intermedia LPS. Thus it led to suppression of NF-kappaB activation. The notion then was proposed that this isoflavone could be used to treat inflammatory periodontal disease. The authors stated that further animal models would be required to be analysed in order to address this aspect for periodontal disease therapeutics [201]. I would add to this and suggest that Amlexanox also may be used to modulate the inflammatory response in periodontal disease – again, animal model systems require to be examined in this regard.

There can be little doubt that uncontrolled inflammation taken in various contexts is harmful despite being part of the body's normal tissue repair and defence mechanism. Periodontal disease offers a good example in this respect – being in a way, an over-reactive response to bacterial pathogenic factors such as LPS. Modulating this via such agents as Amlexanox targeting the proinflammatory NF-kappaB mechanism could well prove useful, as suggested above. So, taken in this direct sense, an example would be involvement of the NF-kappaB cascade in mediating IL-1 stimulation of downstream destructive factors such as Il-6 and MMP-1 in human gingival fibroblasts. IL-1 thus plays a central, direct role in immune-pathological responses of tissue destruction in chronic periodontal disease via NF-kappaB activation [202].

Having said this, one major consideration in terms of periodontal disease is that of smoking. Smoking moderates the NF-kappaB axis and modulates both canonical as well as noncanonical signaling mechanisms. It may be thus said to promote a dysregulated inflammatory response [203]. It remains quite possible that this deregulation may be countered via NF-kappaB axis control, such as with the agent Amlexanox which targets the noncanonical pathway. If this is the case then this agent would then be rather suited in terms of therapeutic prescription for smokers who represent a subset of individuals more prone to periodontal disease – an observation oftentimes noted clinically [204]. A clue that noncanonical kinases are involved in periodontal lesions comes from polymorphism studies [205]. Here, a gene polymorphism of Ikkepsilon has been shown to significantly vary amongst groups of chronic periodontitis vs peri-implantitis individuals. Further investigations are required to visualize exactly what the underlying mechanism for this represents - though it certainly points to the significance of the noncanonical pathway in periodontal disease with its likely associated dysregulation.

Periodontal disease, innate immunity and heart disease

I have already outlined where substitution nonsense mutation in the TLR5 gene (an innate immune receptor; [1]) is a risk factor for diabetes type II – a metabolic disease highly related to periodontal disease [206] and, of course, cardiac and vascular disease with obesity [207]. Amlexanox by targeting such nonsense mutations for readthrough may rescue this phenotype and also aim to correct the insulin resistance and inflammatory-related obesity, as discussed earlier in this article. The point here is made though that innate immunity interference, inflammation and metabolic dysregulation may all walk 'hand-in-hand', thus producing such conditions as diabetes, periodontal disease and heart disease (v.i.).

The periodontal pathogen Aggregatibacter actinomycetemcomitans induces in animal models increases in serum cytokines: Il-6 and TNFalpha. Further, TLR expression is increased within the wall of the aorta. These observations are consistent with the notion that this particular pathogen is capable of accelerating atheroma formation via the inflammatory response [208]. There is also a strong link between P. gingivalis (another key periodontal pathogen) and coronary artery disease (CAD). The mechanism behind this is, in part, via attracting monocytes to vascular endothelium through the TLR2 pathway which mediates LPS signaling [209].

TLR is part of pathogen pattern recognition system in innate immunity. PKC alpha/beta stimulate NF-kappaB via TLR2 stimulation. The PKC complex thus drives LPS-mediated activation of Ikk [210].

Human HSP (heat shock protein) 60 kda and microbial HSP 65 kDa have been implicated in development of chronic periodontitis and CAD. T cells are activated by these HSPs and TLR is recognized by human HSP in both contexts of periodontal disease and CAD. This results in a T cell response to HSP via TLR2 - a shared mechanism between CAD and periodontitis. TLR4 recognised HSP65 of bacteria. A vigorous T cell proliferation accompanying the inflammatory innate immune response may thus be implicated in destructive conditions such as periodontitis and proinflammatory damage leading to CAD [211].

Heart disease – atheroma and NF-kappaB activation

Atherogenesis is related to inflammation which is a key component in its aetiology. NF-kappaB pathway is involved in controlling inflammatory responses, as discussed above, and, as such, is related to atheroma formation. By *in vivo* manipulation of the NF-kappaB signal axis via targeted disruption of Ikkgamma and IkappaBalpha in endothelial cells a reduction in atheroma plaque formation was noted in a high fat diet fed mouse strain. Interference with NF-kappaB reduced cytokine and chemokine production. It also stopped macrophage migration. Thus endothelial NF-kappaB signaling commands proinflammatory gene expression within the arterial wall which in turn may lead to atheromatous plaque development [212].

Interestingly, NF-kappaB is centrally important in diabetes in

respect to CAD. In diabetics, ubiquitin/proteasome hyperactivity is related to enhanced inflammatory response caused by a heightened diabetic oxidative challenge. This in turn leads to increased proteasomal removal of inhibitor to NF-kappaB. Hence this transcriptional factor is more readily available and subsequently diffuses to the nucleus and orchestrates proinflammatory gene cascades. Recruitment of proinflammatory mediators and cells to the athermatous plaque increases plaque instability – key here is quality of the plaque rather than quantity alone. In turn, plaque rupture is more likely in this scenario of decreased plaque stability. Acute plaque rupture is in itself a major cause of atheroma-related serious complications that may present without attendant warning signs such as angina secondary to gradual occlusive CAD. Hence, from this discussion it may be presumed that persons who have type-2 diabetes are more prone to an acute plaque rupture event. Diabetic plaques which were harvested from patients vs controls showed more macrophage activity with T cells and other inflammatory cells. A heightened ubiquitin - proteasome system was noted along with recruited NF-kappaB, MMP9 (metalloproteinase activity) and lower content of plaque supportive collagen [213]. Lower levels of IkappaBbeta were noted as well - this factor being an inhibitor of NF-kappaB. Interestingly, a modified form of sesamol (a natural organic compound and component of sesame oil being antioxidant and a phenolic component of lignans) reduces atheromatous plaque formation via targeting Ikkbeta kinase (a canonical kinase). It thus may effectively moderate NF-kappaB signaling/activation hence reducing atheroma risk [214].

Personal perspective

The discussion in the sections above serve to highlight the connection between inflammation, CAD, the innate immune system/NF-kappaB activation and, as being particularly relevant to myself, periodontal disease with the modifying factor of tobacco smoking which complicates the immune response further. Additionally, metabolic disturbances such as diabetes, commonly present in the general population, seek to further disrupt host response to pathogens and enhance problematic reactivity of the innate immune system and chronic inflammation. Not surprisingly, diabetes is a significant risk factor for periodontal disease as well as CAD. Amlexanox, by moderating the NF-kappaB axis, may well be able to cut the vicious cycle between immune reaction and inflammation – hence, not only positively impacting on diabetes management as has been outlined above, but also in terms of moderating the destructive inflammatory response behind chronic periodontal disease. Ultimately, medical therapies targeting the innate immune system/inflammatory axis may prove much more effective than current therapies used to try to stem the progress of periodontal disease in dental practise.

As a dental clinician I am personally involved with the management of patients with periodontal disease. Oftentimes such persons, particularly those with early onset periodontal destruction, happen to be users of tobacco products. Two key matters need to be addressed in these individuals prone to periodontal disease, viz: excellent plaque (bacterial biofilm) removal and smoking cessation/tapering. Those persons presenting with metabolic disturbance such as diabetes mellitus type II require good control of blood sugar levels along with weight reduction.

Given the significance of dental pathogens on eliciting inappropriate and dysfunctional innate immune response (see above) then clinical interventions with these persons shall prove of little or no value unless combined with strict patient self-removal of plaque on a routine, viz: daily, basis. No element of professional cleaning may compensate for this aspect. Where patients present with stable periodontal condition (viz: nil pain, nor signs of infection or active inflammation with maintenance of supportive bony height and contour over time as monitored via radiographic exam), I discuss home-based plaque care and proceed to place my efforts into restoring their dentition. This latter objective may be performed by means of, for example, fixed bridgework, which has been demonstrated to be beneficial in terms of stabilizing periodontal involved teeth [215]. From the converse, it has been recently stated that periodontal factors have nil mal-effects on bridgework survival over time [216].

One particular caveat deserves mention though, and this relates to patient smoking habits. As I have pointed out [217] tobacco use may potentially mask the cardinal sign of active inflammation, viz: bleeding from the periodontal tissues around the teeth. This may deceive the clinician into believing that the periodontal inflammatory condition is stable, where it may not be. The evidence for this is however somewhat variable in the literature and the astute clinician would do well to use radiographic followup in order to objectively assess whether active periodontal bony destruction, the central feature of this disease, has occurred. Assaying depth of attachment loss around the teeth clinically has worth but may be met with difficulties in assessing small changes over time of soft tissue attachment loss particularly where inflammatory swelling occurs around the teeth intermittently during times of exacerbation. The key objective matter is to radiographically directly assess bony support (quantity and quality) over time.

Further consideration of the relation between periodontal disease and systemic disease is given below in regards the link through inflammation (Concluding discussion).

Figure 8 demonstrates example radiographs (tomograms) of two patients managed for periodontal disease.

Cancer, TBK1, Ikkepsilon and Amlexanox: The prosurvival factor NF-kappaB is related to cancer as I have discussed [39]. Noncanonical kinases too are pro-cancer factors. For example, Ikkepsilon has been noted in itself to impact on cell survival/proliferation [218]. Thus one may label Ikkepsilon as an oncogene [218]. Again, this binds together the notion of inflammation (or the dysregulated control of same) and cancer. As Ikkepsilon and TBK1, the noncanonical activation NF-kappaB kinases, are intimately involved in the inflammatory response, innate immune system and are oncogenic factors, then designing means for regulating these would appear to be a prime focus [219]. It has been recently stated that few targeted inhibitors of these kinases have been identified, at least up to 2012 [219]. A novel high-throughput assay was developed to assist in discovery of small molecular inhibitors of these noncanonical kinases [219]. The aim being to derive new compounds of value to regulate inflammatory diseases and cancer. Recently the discovery of Amlexanox, an FDA approved drug of many years standing, as an effective noncanonical kinase inhibitor (see above) represents a true milestone in the field of inflammatory disease and cancer.

Other studies speak of the involvement of the noncanonical kinases in cancer. For example, Ikkepsilon and TBK1 are capable of activating the prosurvival gene product Akt via phosphorylation reaction [220]. This then relates a mechanism by which the noncanonical kinases may be said to be oncogenic. The noncanonical kinases are intimately connected with malignant transformation [221]. Ikkepsilon is amplified in ~one third of mammary gland carcinomas. Mechanistically, it acts to

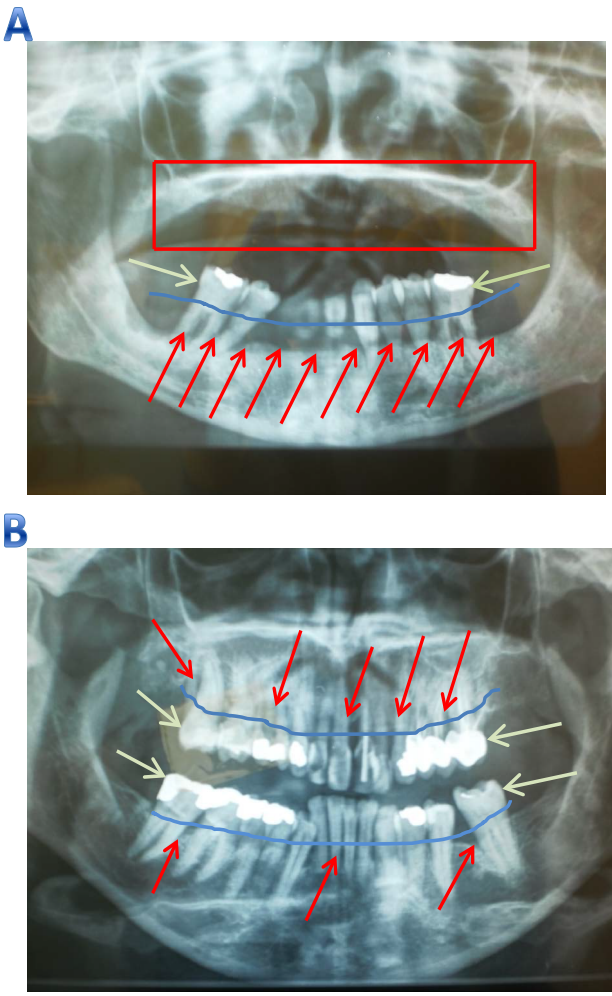

Figure 8: Examples of periodontal disease – radiographic examination.

A) female patient, 65 years old, smoking history, nil heart disease nor diabetes. Areas of periodontal disease for the lower teeth are highlighted with red arrows. The bony tooth support loss is generalised. The upper teeth were lost due in part to periodontal disease (red box).
B) female patient, 63 years old, smoking history, nil heart disease nor diabetes. Areas of periodontal disease are highlighted with red arrows. In this case, the pattern of bony loss is focal rather than generalised.

transform cells via NF-kappaB axis. TBK1 acts as a prosurvival factor and is needed for survival of non-small cell lung cancer as mediated through activated Kras [221]. Clearly, the orchestration of signaling pathways between inflammation and cancer share common features suggesting that control of one may offer up regulation of the other – in other words, they are heavily intertwined at the molecular level. Very importantly, a small molecular inhibitor of the noncanonical kinases such as the long-standing approved agent, Amlexanox, offers potentially a singular major step forward in regulating inflammatory conditions alongside cancer.

Concluding Discussion

The initial purpose of my research was to extend analysis from my earlier investigation [1] showing the important role nonsense mutations play in disease, from cancer to ageing and neurological diseases and many other significant and prevalent disorders. Amlexanox, as a proven efficient readthrough agent has in turn many novel applications in these respects. An account of the impact this already long established therapeutic agent may have overall for communicable and non-communicable diseases is a central feature of my research. Further, a comprehensive examination of the anti-inflammatory properties of Amlexnaox is given. Detailed insights into how Amlexanox 'tweaking' the inflammatory axis via non-canonical NF-kappaB kinase modulation is outlined in regards non-communicable and communicable diseases.

Cancer

Key highlights from this examination of the literature are that there is an interesting alignment of stage of malignancy and nonsense mutation. In other words, nonsense mutations appear to be correlated quite often with aggressive cancer behaviour with spread beyond the immediate primary site. Further, resistance of cancer to therapies may be, in part, due to nonsense mutational alteration.

Certainly, nonsense mutations are at least related within the scope of the 'multi-hit' genetic phenomenon characteristic of cancer development. In liver cancer, [222], substitution nonsense mutations in the TS p53 appeared in clinical stage III/IV cases rather than in those of lower stage. In primary hepatocellular carcinoma (HCC) p53 mutations were found in 51 cases, representing 37%. Four cases carried nonsense mutations. Tumours presenting with p53 mutations were found to be poorly differentiated with advanced stage and poorer outcome. The authors concluded that p53 mutations are common in advanced HCC and occur as late event and correlate with aggressiveness. These p53 mutations did not relate to hepatitis B/C infection [223].

Tumour suppressor p53 mutations are associated with invasive/advanced stage mammary gland ductal carcinoma [224]. Substitution nonsense mutations, viz: p.S297x and p.Y159x in p53 exons 8 and 5 respectively, were isolated in cases from Al-Dakahliya, in Egypt [224]. Authors surmised that such TS p53 mutations seen in that particular locale of Egypt were likely to be responsible for the observed clinical aggressiveness of mammary gland carcinoma.

In advanced urothelial carcinoma of the bladder, genes involved in chromatin modification have been noted to be significantly affected by nonsense mutations [225].

Mutations in the androgen receptor (AR) form a theme in the progression of prostatic carcinoma – PC. Nonsense mutations leading to C-terminal truncated ARs are found at high frequency in metastatic PC [226]. Truncating mutations may act in various unanticipated ways. For example, cells with the AR p.Q640x mutation, are capable of activating AR in neighbouring cells via paracrine effects. This occurs in a ligand-independent fashion and thus demonstrates a certain level of cooperation amongst tumour cells in advanced PC. Further, AR mutations that alter ligand binding and transactivation may facilitate transition of PC from androgen (ligand)-dependent to ligand-independent growth, thus conferring a survival advantage. Substitution nonsense mutation, p.Q640x, was found within the AR in a metastatic PC sample which had escaped androgen deprivation therapy. Notably this mutation is just downstream of the AR DNA-binding domain. This altered AR shows constitutive transactivation properties which could represent a means by which this PC uses to escape androgen deprivation and confer aggressive behaviour [227]. Another study, [228], shows that nonsense mutational inactivation of the PARD3 gene (involved in cell division and cell-polarity determination) in PC LNCaP cell line contributes to PC development and progression.

In colonic carcinoma, ~10% point mutations in exons 5/6 were nonsense mutations. The presence of such point mutations is related to advanced stage cancer and lower survival times in this study from Isfahan, in central Iran [229].

In peritoneal carcinomatosis, there is an extensive spread of tumour within the peritoneal cavity. The primary cancer may source from various locales within the immediate region, viz: pancreas, colon, stomach or ovary. Treatment is a vexing issue and surgery is possible yet limited to highly specialist Centres. Chemotherapy/radiotherapy may also be concurrently performed. Somatic nonsense/frameshift and splice junction site mutations were seen in 39% cases of serous tubal intraepithelial carcinomas (STICs) [230]. The STIC lesion is considered the more frequent precursor to ovarian, tubal and pelvic high-grade serous carcinomas which may develop a peritoneal carcinomatosis phase.

From the above, and from the earlier discussions surrounding the relevance of TS substitution nonsense mutations and tumour development/spread/aggressive behaviour it may be appreciated that rescue readthrough therapy has a vast playing field for cancer.

Further to this, a large part of this present article as indicated has been devoted to outlining the additional side to the readthrough agent Amlexanox, viz: NF-kappaB targeting via noncanonical kinase regulation. NF-kappaB is an important transcriptional factor relating to cancer onset/progression [231]. Earlier drugs such as sulfasalazine, non-steroidal anti-inflammatory agents and glucocorticoids are being actively repurposed for anti-cancer therapeutics [231]. These agents interfere with the NF-kappaB axis. Clearly adverse reactions surround a number of these prior drugs that need to be addressed before they may be used to inhibit their novel outlined target, viz: NF-kappaB.

New drugs have been sought aimed to either inhibit the IKKs or to non-specifically target the proteasome apparatus in order to prevent removal of NF-kappaB inhibitory complex. In terms of the latter, significant clinical information has already been gleaned for Bortezomib, an agent that may be used for multiple myeloma therapy [231].

My aim in this presentation has been to place forward another prior agent, viz: Amlexanox, for NF-kappaB targeting. Given the role inflammation has in cancer then this form of approach with this repurposed drug may prove quite valuable.

An interesting example for where Amlexanox use in targeting noncanonical NF-kappaB axis for cancer may be of value is with Kaposi sarcoma (KS). This cancer is related to Herpes virus 8 infection and is considered a cancer of lymphatic endothelium. It develops as a particularly vascular lesion. G-protein-coupled receptors (GPCRs) are transmembrane proteins that act as signal transducers across the plasma membrane. Herpes viruses encode a number of GPCRs considered to have a role in viral disease. KS-associated herpes virus GPCRs are capable of activating proliferative pathways in endothelial cells such that angiogenic KS lesions evolve. The noncanonical kinase, Ikkepsilon, has been considered related to NF-kappaB modulation in driving inflammatory related diseases, such as cancer. In fact, this kinase is important also in KS-herpes virus GPCR-driven cancer and provides an obvious link between KS-GPCR and NF-kappaB axis. Unsurprisingly therefore, Ikkepsilon is up-regulated in KS lesions and removal of Ikkepsilon abrogates KS formation. KS-GPCR activated Ikkepsilon which in turn led to NF-kappaB nuclear translocation and proinflammatory cytokine expression [232]. These findings link together noncanonical NF-kappaB activation axis with viral encoded G-proteins and the inflammatory cascade to result in productive cancerous lesions of KS. Amlexanox, by targeting Ikkepsilon, [180], may well be able to break this link or vicious cycle and hence therapeutically intervene to prevent KS development.

Overall, it has been reviewed recently that oncogenic viruses have been associated with inflammation via the NF-kappaB signal axis amongst other key pathways [233]. This inflammation in turn promotes an environment suited for cancer development. Chronic inflammatory conditions such as periodontal disease have also been associated with increased cancer risk [234,235]. Perhaps this is not entirely surprising considering that periodontal pathogens relate to dysregulation/activation of NF-kappaB – a central regulator in inflammation [236] (Sections: 3.10.3.3.1 Periodontal disease and TBK1; 3.10.3.3.2 Periodontal disease and Ikkepsilon). Cancer promotion aligns with this mechanism also (Cancer, TBK1, Ikkepsilon and Amlexanox). Overall, this is of considerable interest as it reflects an important 'bridge' or connection between communicable and non-communicable chronic diseases (heart disease, cancer, diabetes, rheumatoid arthritis, asthma, neurodegenerative conditions) in regards pathogenesis (see below). Indeed as it has been stated in regards identifying NF-kappaB as a central effector of inflammation: "...makes a possible way for the development of new therapeutic approaches using synthetic and natural compounds" [236] which would be in turn aimed to decrease prevalence of chronic non- communicable diseases. Amlexanox is the focus in terms of such a novel agent with my present article.

To date, therapeutic agents such as Sulfasalazine and Rebamipide have been used as inflammation modifiers via the NF-kappaB axis. Sulfasalazine is an FDA approved category B pregnancy drug (as is Amlexanox) used to manage inflammatory conditions such as rheumatoid arthritis, ulcerative colitis and Crohn's disease. It reduces the synthesis of inflammatory cytokines via inhibition/modulation of NF-kappaB [237]. In this way it has benefits in terms of management of ulcerative colitis, an inflammatory condition that, if extensively presenting, may enhance the risk for colonic cancer. Again, tying together the notion of chronic inflammation acting, at least in part through the NF-kappaB axis, as a carcinogenic risk factor.

Rebamipide is a quinolinone derivative that is used extensively in Asia for gastrointestinal ulceration – though not FDA approved for use in America. Interestingly, Rebamipide mechanistically acts to suppress the TLR/TBK1 signaling platform [238]. This ties together anti-inflammatory therapeutics with the innate immune system. Rebamipide was found to reduce expression of the TBK1 noncanonical kinase and IRF3/7 induced via LPS (bacterial pathogen indicator) and dsRNA (viral pathogen marker). As TBK1 is required for production of inflammation in patients with ulcerative colitis then Rebamipide ought to form a suited targeted therapeutic approach to this potentially precancerous inflammatory condition, as the authors propose.

Clearly, agents such as Amlexanox which also moderate the NF-kappaB signaling axis through noncanonical kinase interaction could be well posed for adding to the general armamentarium against destructive inflammatory conditions. Given that both Sulfasalazine and Rebamipide appear to be relatively well tolerated it would support the notion that chronic therapeutic intervention at the level of NF-kappaB does not seem to carry significant adverse reaction.

So, aside from the readthrough capabilities of Amlexanox, the

worth of this repurposed agent may also be found through intervening with the biologically very important NF-kappaB signal axis, particularly in regards many chronic conditions, including cancer.

Cardiac and vascular disease

Heart disease and inflammation – periodontal disease: I have already adequately presented where substitution nonsense mutational rescue may have a significant place within the field of heart diseases [1]. Having said this, being a dental surgeon, I am keenly interested in dental health and its impact on general – systemic health matters. One prime example here is in regards the cardiac and vascular system and periodontal disease, a very prevalent dental condition Worldwide, affecting up to ~one third of adults to varying degree. Periodontal disease is essentially a destructive inflammatory-based condition secondary to bacteria and their products (Periodontal disease, Ikkepsilon, TBK1 and heart disease). As discussed above, such factors as the outer membrane protein MspTL present on T. lecithinolyticum (a pathogen associated with periodontal disease) may induce host proinflammatory cytokines such as CXCL10 and activates the TBK1 noncanonical kinase signaling axis [198]. Notably, Daidzein an isoflavone found in plants, inhibits LPS of the periodontopathic bacterium P. intermedia from inducing interleukin-6 and other proinflammatory messengers and prevented NF-kappaB activation. Clearly this compound may be beneficial in managing periodontal disease [201].

The NF-kappaB cascade mediates IL-1 stimulation of destructive factors such as Il-6 and MMP-1 in chronic periodontal disease [202]. The presence of the periodontal pathogen Aggregatibacter actinomycetemcomitans leads to increased cytokine levels of: Il-6 and TNFalpha and is capable of accelerating atheroma formation via inflammatory response [208]. P. gingivalis, a noted periodontal pathogen, is also linked to CAD [209] (Periodontal disease, innate immunity and heart disease).

Atherogenesis is related to inflammation which is a central factor in its aetiology with the NF-kappaB pathway being key in controlling inflammatory responses (Heart disease – atheroma and NF-kappaB Activation).

Therefore, noting the above discussions, it is fairly clear that there would be anticipated, from first principles, to be a relationship amongst the variables of NF-kappaB signalling axis, inflammation and atheroma formation/CAD. Indeed, clinically, as mentioned, this may be supported, viz: periodontal disease relating to cardiovascular disease risk [197]. In fact, all indicators would suggest that there would appear to be a good argument in support for an unhealthy connection between chronic low grade unresolved inflammation from the periodontal tissues (which covers a significant extended surface area), immune axis signaling, cytokine production and reactive vascular disease (CAD). Many risk factors may predispose to CAD that oftentimes appear in persons with periodontal disease. These may be intrinsic factors such as diabetes with poor weight control or extrinsic factors such as smoking (Personal Perspective). These cofactors/confounders need to be assessed and appropriately controlled for prior to passing judgement on a direct cause-and-effect relationship between periodontal dental disease and cardiovascular pathology. The status quo on this contentious subject has been neatly phrased recently: "Observational studies to date support an association between PD (periodontal disease) and ASVD (atherosclerotic vascular disease) independent of known confounders. They do not, however, support a causative relationship. Although periodontal interventions result in a reduction in systemic inflammation and endothelial dysfunction in short-term studies, there is no evidence that they prevent ASVD or modify its outcomes" [239]. In order to answer why this direct cause-and-effect relationship is rather blurred (that is, PD is not apparently related directly to ASVD), I propose the following novel concept in the discussion below, which is important when it comes to seeking therapeutic intervention such as via NF-kappaB axis targeting.

Explaining the uncertain cause-and-effect relationship between periodontal inflammatory disease and vascular disease – the prosurvival model and the cardiac and vascular system - two sides of the one coin: As my research has spanned both cancer and cardiac and vascular disease, I have constructed a unifying model (the prosurvival model, [39]) that matches survival pathways between heart and cancer. This model points out that many factors such as the NF-kappaB axis (relating to cytokine production) along with cytokines themselves such as CX3C along with many other pro-growth factors like VEGF are in fact dual cardioprotective and cancer prosurvival factors. This is in fact a general, unifying concept that may be taken across biology as a whole, for example, extending the concept to neuroprotection, and other organs/tissues. Further, because of this association, I may state that cancer related biomarkers in fact mimic cardioprotective markers and aid in determining cardiac and vascular risk assessments [240]. Additionally, as I have importantly indicated of late, the cardiac and vascular system although acting as parts of a whole, is in fact functionally partitioned [241]. For example, proinflammatory cytokines such as interleukin-6 and TNF are on the one hand cardiocytoprotective yet have adverse effects on the vasculature [241].

Appreciating these novel outlined principles may well prove to be the key in understanding the apparent ambiguous link between periodontal inflammation/disease and cardiovascular disease – mechanistically to date an unsolved conundrum to the dental-medical community. The key concept is that cytokines, which are oftentimes considered the 'culprits' produced from inflamed tissues (see above) have two sides or a duality to their nature – a Jekyll and Hyde characteristic. For example, a well featured cytokine, interleukin-6, is not only pro-inflammatory and correlated with periodontal disease and damage [242] but also has prosurvival (cytoprotective) capacity towards cardiomyocytes [243] – as I have further recently stated [241]. In accord with the generality of the prosurvival model (as indicated above), this cytokine is hepatocytoprotective [244] and neurostimulatory/protective [245,246].

A master regulator of cytokine production, NF-kappaB, relates significantly to cytoprotection, as I have amply discussed previously through my 'prosurvival model' [39]. Notably, this 'master' transcriptional factor is cardioprotective [39] as well as regulating a key survival cascade in cancer. Having said this, an emerging central axis in periodontal inflammation is activated NF-kappaB and ways by which this signalling axis may be put under tether or control would appear to be attractive in better managing periodontal disease [247]. General blockade of NF-kappaB is not desirable of course due to its pluripotent cytoprotective properties [247], but modifying or 'tweaking' its activation via specific targeting such as with Amlexanox therapy (Amlexanox and innate immunity) may well be a suitable means of approach (Implications for therapeutic intervention).

Another interesting factor is caveolin-1, a protein that aids scaffolding in the caveolae membranes within cells. These form as small invaginations into the plasma membrane of endothelial cells and adipocytes in particular. They may function in signal transduction,

endocytosis and pathogen response. Caveolin is a prosurvival factor shared between heart and cancer, as I have indicated [39]. Interestingly, Caveolin-1 has been shown to induce new blood vessel formation – angiogenesis [248] in the context of inflammation. A significant level of Caveolin-1 is seen in periodontal disease lesions and was responsively produced by TNFalpha and interleukin-1beta stimulation in periodontal fibroblasts. Caveolin-1 increased metalloproteinase and VEGF production in periodontal cells. These data indicated that Caveolin-1 is capable of enhancing the destructive, inflammatory response in periodontal disease [248]. The pathway employed by Caveolin-1 was MAPK – a leading prosurvival path for heart and cancer in its own right [39]. Further, VEGF is also a matching prosurvival factor [39]. These findings complement nicely my prosurvival model concept for inflammatory pathways linking between cardiomyocyte cytoprotection on the one hand and destructive inflammatory lesions, viz: periodontal disease, on the other.

The chemokine, CX3CL1 (fractalkine), is elevated in the cardioprotective reactive process, hibernating myocardium, and in malignant gliomas. I have thus considered fractalkine as a prosurvival factor [39]. This chemokine and its receptor are implied to have a role in enhancing leucocyte migration to inflammatory periodontal disease lesions [249]. As such, it most likely promotes the periodontal destructive process. Again, unsurprisingly in terms of applying my prosurvival model to explain the mixed reaction of the cardiovascular system to progressive periodontal disease.

NF-kappaB and interleukin-6 and other cytokines/factors have been amply discussed in regards periodontal disease in this article (Periodontal disease, Ikkepsilon, TBK1 and heart disease). It has also been outlined that cytokines and inflammatory pathways may mediate atherogenesis leading to CAD (Heart disease – atheroma and NF-kappaB activation). So the situation is that leading proinflammatory regulators such as NF-kappaB and individual cytokines are not only necessary for cytoprotection sparking cardioprotection, but are also a double-edged sword, leading to inflammatory-related vascular disease. A nett effect is what one is probably observing clinically of the opposing arms of this dual-sided nature of cytokines and their master regulators. In that sense, the relation between chronic states such as periodontal inflammation and cardiovascular disease is blurred, ambiguous and lacking a clear cut outcome.

Amlexanox and the heart: It would be interesting to investigate whether Amlexanox, as an agent capable of modulating NF-kappaB activity, is also able to have a directly beneficial effect on the heart itself. Already it has been recently shown that the agent IMD-1041 may be able to prevent pressure overload-related cardiac malfunction which leads to heart failure via suppression of NF-kappaB activation [250]. Further, cardiac inflammation related to NF-kappaB activation in cardiomyocytes is considered a feature of heart failure [251]. Having stated this, very recently it has been shown that the non-canonical NF-kappaB signaling axis is also related to cardiac health [252]. Deficiency of ikkepsilon, a non-canonical NF-kappaB kinase and Amlexanox target, leads to hypertrophic alterations probably by unbalancing the NF-kappaB prosurvival homeostatic mechanism in heart cells. Although I have clearly stated that NF-kappaB is in fact a cardioprotective factor [39] there is a clear balance that needs to be struck in terms of its expression/activity that reflects the overall importance of homeostasis in cardiac physiology [241]. In effect, too much of a 'good thing' (viz cardioprotective factor expression such as NF-kappaB) may prove 'not so good'. Due care to the cardiologic effects Amlexanox may have in the longer term need to be considered and followed-up with appropriate heart function testing (echocardiography for example) in patients who would be selected for chronic dosing with this therapeutic. Despite that caveat, it appears from the evidence to date that Amlexanox has not raised any material concerns with respect to cardiology. Again, speaking of the safety margin that appears to be present despite modulation of NF-kappaB activity.

Bone homeostasis/physiology, cytokines, periodontal disease and arthritis

The relation of truncating mutations and bone disease has been presented (Bone disease and nonsense mutations). Bone homeostasis, viz: the balance between osteoclastic bone resorption vs osteogenesis, is complex. Nonetheless, this is highly relevant to inflammatory states such as periodontal disease as bony loss of tooth-support is a central feature of that condition. A number of important cytokines signal via the gp130 receptor complex [252] including interleukin-6/11 and cardiotrophin-1. These contribute to inflammation and maintain bone homeostasis. They have a role also in pathology involving calcified tissues in periodontal disease and rheumatoid- and osteo-arthritis. Osteoclast cell (resorptive) formation from mononuclear precursors is stimulated via gp130-related cytokines such as interleukin-11, interleukin-6 and cardiotrophin-1. Interestingly, interleukin-11 is another cytokine related to cardioprotection [253,254] and, along with interleukin-6 and interleukin-1beta, interleukin-11 was significantly elevated in periodontal disease [255]. Interleukin-11 is involved in bony resorption in cancer [256] and this has implications in regards its likely role in bony destruction in periodontal disease. The role of the endocrine system via parathyroid hormone (PTH) is significant too as PTH stimulated osteoclast formation through interleukin-11 and interleukin-6 receptor [252].

The duality of interleukin-11, as with other cytokines, in promoting cardioprotection (cardiomyocyte cytoprotection) may well compensate for its pro-inflammatory effects on the vascular system. This is a feature of interleukins and cytokines overall that I have recently noted [241].

Another key dual-acting pro-inflammatory cytokine is interleukin-1. This also results in osteoclast formation via the gp130 pathway [252]. Interleukin-1beta activates the NF-kappaB cytoprotective signal pathway in cardiomyocytes [257] along with interleukin-10 [258]. Interleukin-1beta is notably elevated in periodontal disease [255,259] along with interleukin-10 [260]. Therefore these data provide further evidence for the competing properties of cytokines, viz: one side pro-inflammatory destructive and the other being cytoprotective [241].

Cardiotrophin-1 cytokine is an interleukin-6 family member. Interestingly, it acts via NF-kappaB activation to mediate cardiomyocyte cytoprotection [261]. It exerts its effects through interacting with the gp130 joint receptor complex [252]. Although a defined role for cardiotrophin-1 in periodontal disease is presently not clear. Nonetheless, it may be importantly involved in bone homeostasis as null cardiotrophin-1 animal models show impaired osteoclast (resorptive) functioning [252]. In this respect, any possible role in periodontal disease may well follow suit with other related cytokines. Interleukin-6 itself shows an overall inhibitory role in bone formation consistent with its place in periodontal bony resorption. Further, lipopolysaccharide from bacterial pathogens stimulates interleukin-6 release in periodontal ligament cells [252]. Another inflammatory cytokine correlating with interleukin-6 production in periodontal disease is interleukin-8 (a chemokine produced by macrophages and

endothelial cells, [242]). It should be noted that interleukin-8 has also been implicated in cardioprotection [263].

The relevant bone biology of receptor activator of nuclear factor-kappaB ligand (RANKL), RANK and osteoprotegerin axis is valuable to consider [262]. RANKL cytokine is found on osteoblast surfaces and activates osteoclastic cells by interacting with RANK [263] on the latter cell's surface. Notably, pro-osteoclastic gp130 cytokines increase osteoclast formation via acting on precursor lineage cells to increase the key osteoclastic (resorptive) factor, RANKL, and reducing its inhibitor osteoprotegerin (a decoy receptor which binds RANKL inhibiting NF-kappaB activation). RANKL is produced in periodontal fibroblasts as regulated via pro-inflammatory cytokines [264]. Thus the RANKL/osteoprotegerin balance may be predicted to be important in modulating periodontal bony resorption. NF-kappaB axis being at the root of this complex signalling system. Agreeing with the above concept relating duality of cytokines and cardioprotection it is interesting to note that osteoprotegerin, an inhibitor of RANKL, has been found to correlate with heart disease [265]. This suggests that RANKL is a cardioprotection-related mechanism in its own right. Supporting this notion is the observation that optimal osteoclast formation driven by RANKL signalling may also require added factors such as the cardioprotective cytokines cardiotrophin-1 and interleukin-11 via gp130 signal pathway [252].

Figure 9 illustrates the dual prosurvival/cytoprotective vs proinflammatory/damaging nature of cytokines produced by inflamed tissues such as found in chronic periodontal disease.

No presentation regarding inflammatory regulation of bone homeostasis would be complete without some mention of arthritic conditions. Both rheumatoid and osteoarthritis have a significant destructive inflammatory component. In rheumatoid arthritis, the cytokine 'soup' at the front of disease progression promotes RANKL production and decrease in inhibitory osteoprotegerin [252]. This in turn promotes osteoclastic (resorptive) activity. Other key cytokines mediating damage in RA include interleukins-6/11. In many ways, RA mimics periodontal disease in respect to the damaging influence of inflammation. TNFalpha is a key pro-inflammatory cytokine driving inflammation linked tissue damage in RA and ulcerative colitis. A mechanism of action is via the noncanonical signalling axis of NF-kappaB activation [266]. TNFalpha along with interleukin-6 has a significant role in destructive periodontal disease in the context of obesity which in itself relates to diabetes and CAD [267]. Clearly, any drug that relieves obesity or targets the signaling axis of TNFalpha could be predicted to be a very useful addition to the therapeutic armamentarium against a host of illnesses, including periodontal disease, CAD/vascular disease, obesity and diabetes. My proposal is for the use of Amlexanox as such an agent (Implications for therapeutic intervention).

Implications for therapeutic intervention - other inflammatory related diseases

It has been already suggested that by specific targeting of key host signal pathways - such as NF-kappaB, inflammatory-related diseases may be able to be bridled [247]. Periodontal disease, RA, and other very significant inflammatory-related conditions, such as obesity-related insulin resistance, require careful and tight modulation of the inflammatory signaling axis. In periodontal disease, a mechanism by which the vicious cycle of NF-kappaB activation via pathogen exposure needs to be 'cut' as it were. Self-sustaining inflammatory circuits in other chronic conditions such as obesity/insulin resistance and RA need to be also 'disconnected'. From the above, it may be seen that Amlexanox, by moderating the NF-kappaB response, may well be able to provide a disconnection in the vicious cycle of chronic inflammatory conditions. My personal interest is within the scope of periodontal disease. In this field, Amlexanox may well offer considerable hope for a medical therapeutic approach that has not been able to be achieved before. Only by improved understanding of the molecular biology of the host-pathogen response through reactive inflammation is it at all possible to conceive this approach. Targeting the noncanonical NF-kappaB axis may, on the one hand, reduce destructive inflammatory response, yet on the other, could affect host response to pathogen exposure at the site of infection (Bacteria and noncanonical NF-kappaB kinases, [194]). Clinical trials are called for, yet in the ultimate, adequate patient home care and cessation/tapering of the significant co-factor in periodontal disease, viz: smoking, (Personal Perspective) would be predicted to be very important influences in regards outcome of drug-based (Amlexanox) anti-inflammatory therapies.

Many neurology-based illnesses and conditions may be classified as genetically based, a viewpoint I have outlined already in terms of readthrough rescue therapy (Readthrough nonsense targets: Neurological illness). In addition, as a causative feature in these disorders, neuroinflammation has been suggested to play a significant role. This is certainly the case for neurodegenerative conditions such as Alzheimer disease and Parkinson disease [268]. A potent aspirin derivative has been shown to have anti-inflammatory and neuroprotective features and reduces interleukin-6 and TNFalpha cytokines with concomitant reduced activation of NF-kappaB in neural-type cells [268]. Anatabine, a minor tobacco alkaloid is anti-inflammatory and inhibits NF-kappaB activation by bacterial lipopolysaccharide (LPS) [269]. This compound also reduces pro-inflammatory cytokines such as interleukin-6, 1beta and TNFalpha in animal models challenged with LPS. Further, this anti-inflammatory reduces brain TNFalpha and interleukin-6 in a mouse model for Alzheimer neuroinflammation.

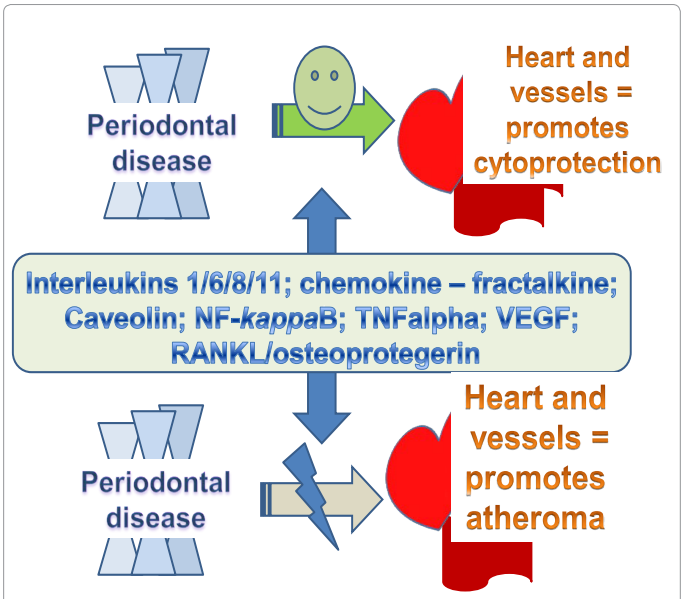

Figure 9: Cytokines produced by inflammatory tissues, such as periodontal disease illustrated here, have two sides – a prosurvival/cytoprotective *vs* damaging/proinflammatory nature. These two opposing sides act in balance and competition to each other. Atheroma is shown as an example of inflammatory disease related process.

Obovatol from the medicinal herb, Magnolia obovata, possesses anti-inflammatory properties [270]. By artificially activating NF-kappaB axis in neural cells proinflammatory factors were released such as TNFalpha and interleukin-6. Pretreatment with obovatol reduced this effect and appeared neuroprotective [270]. This indicated that such medicinal therapies may reduce the destructive neuroinflammation that is evident in such conditions as Alzheimer and Parkinson disease as well as amyotrophic lateral sclerosis.

The downstream adaptor of TLR3 – TRIF - forms a major part of the innate immune system signalling axis that leads to neuroinflammation in the brain [271]. Overproduction of TRIF may consequently lead to hyperactivation of the pro-inflammatory response in the central nervous system. This in turn may be responsible for neurodegenerative conditions. Knockout TRIF animal model demonstrated reduced inflammatory cytokine levels with reduced activation of downstream effectors such as TBK1, IKKepsilon and NF-kappaB were reduced significantly [271]. Furthermore, optic nerve axon regeneration was supported in the TRIF knockout model suggesting that by reduction of NF-kappaB activation and noncanonical kinase signalling pathways then adverse neuroinflammatory cytokine levels are suppressed. This in turn was neuroprotective.

Clearly, in neurology, Amlexanox therapeutics may find a niche in terms of significantly impacting on neuroinflammatory-related conditions – a growing field of interest in its own right.

Figure 10 shows the potential application of Amlexanox therapy for a variety of inflammatory-related conditions.

Managing Ageing as a disease: Targeting nonsense mutations and inflammation - Enter Amlexanox

The intriguing concept of viewing ageing as a disease enables a potentially very tangible therapeutic approach to what has been to date commonly considered a 'natural process'. In a sense, one may extend this viewpoint by examining ageing as a 'stage' of life much as one would view a particular stage of any disease process.

Premature protein truncation and ageing: A prime focus of this article has been to outline where nonsense mutations align with clinical stage, for example in cancers. In much the same way, nonsense mutations oftentimes align with early onset or increased stage of a host of other conditions from osteoporosis to neurodegenerative conditions as I have presented in Results and General Discussion (Figure 11). Further examples may be presented along these lines. Early onset macular degeneration/atrophy has been linked to premature truncating mutation in PROM1, a five-transmembrane glycoprotein [126]. Other studies have also linked various causative genes presenting with premature truncating events to early onset macular pathology [272,273]. Early onset severe or advanced stage renal dysfunction with cardiac hypertrophy and hypertension, a format of signs and symptoms that one may find in advanced age, was found causatively related to nonsense mutational change in WT1 [274]. This mutation deleted WT1 zinc finger 3 domain. Notably, WT1 is a factor required for normal heart and kidney development and is also involved as a TS [275].

Inflammation and ageing: Resveratrol has cardioprotective and anti-cancer properties as I have discussed [39]. Its anti-oxidative, anti-inflammatory and anti-ageing capabilities have been well noted [276]. This broad and beneficial spectrum of activity aligns with Resveratrol being able to suppress TRIF and TBK1 which correspond with NF-kappaB modulation as well as AP-1 and IRF-3 inhibition [277]. Amlexanox also is a modulator/inhibitor of TBK1 and hence is involved with the TRIF/IRF3 pathway. Thus, it would be evident that Amlexanox is capable of readily 'following in the footsteps' of Resveratrol as an anti-ageing therapeutic targeting the non-canonical branch of the NF-kappaB inflammatory axis. The above quoted Resveratrol investigation is very significant in respect to Amlexanox since, logically, as Resveratrol is commonly consumed not just in red wine but also in tablet form over-the-counter (in Australia) it carries nil obvious adverse effects. This supports the general prescription and safety of Amlexanox since this agent as mentioned also targets the TBK1 axis of NF-kappaB. Certainly concerns regarding alteration in innate immune response may be lessened when considering the Resveratrol studies and is consistent with the concept that modulating NF-kappaB is relatively safe at therapeutic levels.

Inflammation links ageing to the neurological system [278]. This is not surprising as inflammation is a mark of age-related processes

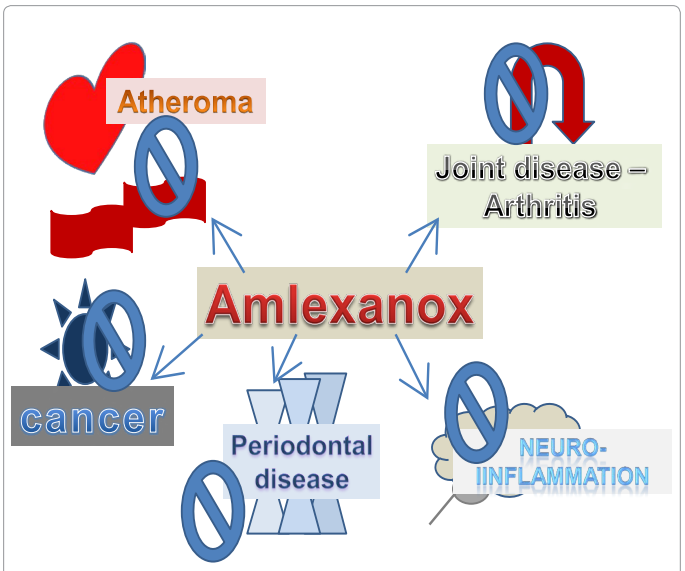

Figure 10: Proposed uses for immunomodulatory agent such as Amlexanox for inflammatory-based diseases.

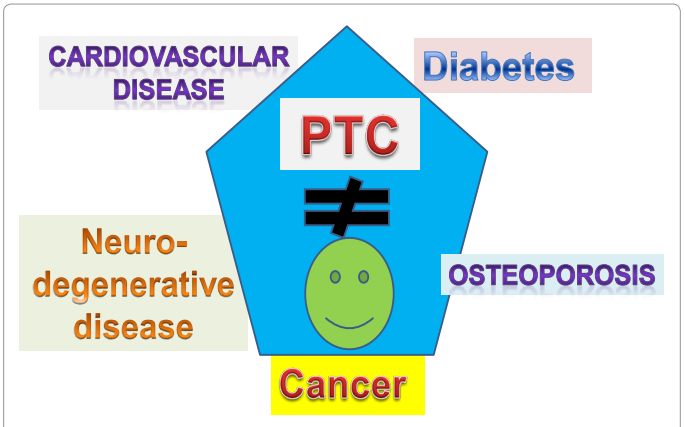

Figure 11: Early onset conditions – reflecting premature ageing – may be related to nonsense mutations. These include neurodegenerative conditions (dementias), osteoporotic disease, early onset type II diabetes and, more broadly, cardiovascular illness and cancer.

such as diabetes, cardiac and vascular disease, arthritis and Alzheimer's and probably I would also say such chronic conditions as periodontal disease. This connection relating to ageing and inflammation is termed: inflammaging [278]. NF-kappaB signaling axis is a key player in the brain during ageing [278]. In fact, the hypothalamus has been outlined to be a central factor related to activated NF-kappaB and systemic ageing [278]. Clearly, any anti-inflammatory agent that specifically targets NF-kappaB and is capable of entering the central nervous system could well impact on systemic inflammaging. I propose Amlexanox be examined in this respect – this agent could well prove to be a significant modulator of central inflammation that would have far reaching effects. Already in animal models Amlexanox is able to beneficially moderate metabolic inflammation and reduce obesity and insulin resistance, hallmarks of diabetes [180].

The above analysis thus opens up a novel therapeutic vista in respect to actually 'treating' the ageing process at a Personalised Medicine level. I propose use of Amlexanox for this purpose given its readthrough efficiency, anti-inflammatory properties and safety record over 2-decades. The technology of Amlexanox readthrough would appear safe and straightforward - important in terms of patient compliance. Other techniques such as stem cells and approaches aimed at modulating the NMN (nicotinamide mononucleotide)-NAD+ pathway for ageing have gained much recent attention [279]. Although it must be pointed out that the latter concept involving NMN has already been revealed several years ago in regards age-related (late onset) diabetes [280]. These approaches remain untested for longer term safety whereas Amlexanox already has an extensive >20-year record for clinical use [1] and is relatively inexpensive and readily compliant for direct patient application. Amlexanox also has a multitasking potential targeting nonsense mediated changes relating to ageing (modeled as a disease or 'stage') alongside its effective targeted anti-inflammatory properties. The time would therefore appear just right to repurpose Amlexanox for anti-ageing strategies.

Amlexanox, nonsense mutations: NMD and animal models

As previously mentioned, Amlexanox has a chemical weight and size similar to Ataluren, another small molecular weight readthrough agent [1]. The precise mechanism by which Amlexanox effects readthrough is not known, but clues on this aspect derive from other studies examining its action in terms of NF-kappaB non-canonical kinase targeting [177]. In this study, Amlexanox is demonstrated in modeling analysis as binding to the ATP site of TBK1. A Lineweaver-Burk plot showed Amlexanox competition with ATP for inhibition of ikkepsilon [180]. These data may support the notion that Amlexanox is capable of mimicking ATP interaction with protein(s) of the ribosomal complex and effecting an allosteric change that alters the conformation of the decoding site of the ribosome. More studies are called for to explore the precise mechanism of readthrough activity and whether the notion of ATP-interaction and allosteric changes are involved.

Amlexanox has also the property of inhibiting NMD [1], yet again, the precise mechanism for this is unknown. It has been indicated that this inhibition may occur late in the NMD pathway [281]. Importantly, Amlexanox did not alter the phosphorylation status of eIF2alpha [282]. This factor has been observed to be phosphorylated as part of the NMD inhibition pathway [283]. Cancer uses this eIF2alpha-phosphorylation mechanism to program its microenvironment in order to support its growth and cell survival [284]. Reassuringly, Amlexanox does not appear to participate in this specific process for NMD inhibition but uses alternative routes.

Disease and nonsense mutations really do walk 'hand in hand' – a central feature of this and my previous article [1]. Smith-Lemli-Opitz syndrome (SLOS) is an interesting condition where there is an inborn error of metabolism, viz: cholesterol synthesis. Mutation of DHCR7 (7-dehydrocholesterol reductase) is causative. The phenotype includes ID, behavioural problems including aggressive personality and numerous organ defects. Notably, cholesterol cannot cross the blood brain barrier and therefore needs to be made within the central nervous system. As cholesterol is a component of cell membranes it comes as little surprise that SLOS patients have a significantly disturbed neurological physiology. Nonsense mutations in DHCR7 resulting in SLOS have been outlined. NMD of the identified p.Q98x and p.W151x alleles was also determined [282]. Readthrough agent G418 may also suppress NMD to some degree (although is not a viable readthrough agent due to adverse toxic effects longer term). This agent increased cholesterol production from the p.Q98x cell line but was ineffective for the p.W151x allele [282]. Low efficiency in readthrough of this allele due to codon context as well as critical residue substitution may account for these results. Amlexanox, being an efficient NMD inhibitor and capable to rescue stop codons in a variety of contexts may well prove to be a more suited drug to use in correcting this debilitating inborn error of metabolism. Rescue of residues that demonstrate a critical amino acid (active site components for example) may prove more challenging [1]. Only by experimental testing may one be able to come to conclusions in this regard. Importantly too, in regards SLOS, is the provision of therapeutics that are able to cross the blood brain barrier to enable cholesterol synthesis to be achieved within the brain. It is uncertain to what degree Amlexanox is able to negotiate this barrier [1]. Further studies are called for in this regard. Another important neurological condition already discussed in the context of readthrough is Rett syndrome [1]. The nonsense mutation, p.R270x, is one of the most frequently found mutations in MECP2 resulting in Rett syndrome in Australia. Importantly, this particular nonsense mutation leads to a more serious presentation with decreased survival [282]. Clearly, any readthrough agent that could be applied to rescue this mutation in an efficient and non-toxic fashion given early after birth or even prior to birth would be hugely welcomed not only in Australia but around the World. In this respect I propose Amlexanox for trialing with such nonsense-mediated Rett syndrome cases.

Another important inborn error of metabolism is phenylketonuria. Readthrough *in vitro* with aminoglycosides G418 and gentamicin for nonsense mutations in the causative gene, phenylalanine hydroxylase, has been achieved [28]. The readthrough product was capable of restoring some degree of enzymatic activity, though not to wild-type levels. Authors concluded that this investigation offers a proof of concept in terms of management of nonsense mediated inborn errors of metabolism. Application of the already safely determined and efficient readthrough drug Amlexanox to clinical trials for phenylketonuria and many other nonsense-related inborn errors of metabolism is a proposition I make in this article.

Preclinical studies using animal models are highly valuable. For example, a mouse model for early-onset renal failure as caused by a nonsense mutation in xanthine dehydrogenase is available and would form an excellent resource to study Amlexanox as a rescue agent for renal disease in humans [283]. The Nur7 mouse is a result of a nonsense mutation in the aspartoacylase gene and this produces a spongy degeneration of the central nervous system [285]. In humans, deficiency of this enzyme leads to a fatal leukodystrophy, viz: Canavan disease. The mouse model ought to be used to investigate the use

of Amlexanox for targeting this disease, specifically the nonsense-mediated human counterpart. The zebrafish too forms an excellent resource for preclinical studies of nonsense-mediated disease. In regards inherited eye diseases there is a large degree of genetic variation. Nonetheless, an underlying feature at the molecular genetic level in a significant proportion of these conditions is nonsense codon mediated [286]. As the authors stated: 'A therapeutic intervention targeted at this abnormality would therefore potentially be relevant to a wide range of inherited eye diseases'. Aminoglycosides were used in the zebrafish model of several eye conditions and restored functional proteins [287]. These significant studies point to the utility of readthrough for human eye diseases and it is my proposal to use Amlexanox not only in such preclinical models but also for clinical trials in patients with eye conditions. The preventive nature of readthrough in the zebrafish model suggests that this technology may also be applied early in development. Use of a safe agent such as Amlexanox would appear ideal in this respect. Post-developmental correction of such defects would have to be assessed also through appropriate trials.

Amlexanox, due to its anti-inflammatory as well as readthrough capabilities may be well suited for skin cancer prevention. Many skin cancers and precancerous conditions present with nonsense mutational alterations in TS loci [1]. Further, it is evident that inflammation is a key feature in the development of skin cancer. Animal models representing this relationship offer an ideal opportunity to test the potential benefit of Amlexanox as an anti-inflammatory preventive agent for skin cancer. The notion of combining anti-inflammatory drugs with UV-blocking agents in sun lotions would be a very appealing proposition given the involvement of inflammation and UV-radiation in determining skin cancer risk. I certainly would support the use of approved anti-inflammatory agents such as Amlexanox for this purpose.

Final comments

At present there is a large focus being placed on the development of safe and efficient readthrough agents [288]. My proposition aims to place forward into the spotlight Amlexanox. This therapeutic has had a long history of clinical acceptance from mouth ulcer management (in America) to asthma management (in Japan). Amlexanox truly possesses some very unique and highly sort after qualities. It is able to demonstrate efficient readthrough of various codons in varied contexts. Further, it inhibits NMD – a feature needed to efficiently drive the readthrough process – necessary for the rescue of the myriad of nonsense-mediated illnesses/conditions/syndromes that may be diagnosed via genetic technologies. It has another side to its nature also – immune modulation. This it achieves via controlling the activation of the noncanonical NF-kappaB kinases, Ikkepsilon and TBK1. By regulating these inflammation may in turn be controlled and potentially the consequences of same, such as obesity-related diabetes, cancer and immune-related conditions such as arthritis amongst many other conditions. In many ways, Amlexanox appears as an 'all-round' panacea. One concern is that by immune-modulation, long term Amlexanox exposure may alter host response to pathogen challenge. In Japan, Amlexanox has been approved for many years for asthma management [289-291]. Indeed, treatment p.o. at 150mg/day Amlexanox was beneficial in moderating asthma over an extended period of up to eight months [290]. Amlexanox has a structure reportedly not unlike that of sodium cromoglycate (IntalTM), a mast cell stabilizer and anti asthmatic medication [291] and this may account for some of its beneficial effects in regards the respiratory system. Like Amlexanox, cromoglycate is pregnancy category B. Certainly these studies make the point that long term sustained dosage of Amlexanox does not appear to be in any way harmful.

In summary, this article aims to provide a broad introduction to appreciating the considerable potential that Amlexanox holds. This agent has dual properties, making it attractive as an 'all-purpose' mono- or combined therapy for a whole range of conditions. Readthrough alone has a very bright future with a vast repertoire of targets at one's disposal. Recently, Ataluren, a useful second generational readthrough agent [1,23] has shown great potential for clinical application to pulmonary arterial hypertension (PAH) [292]. This is a serious disease of the lungs and ~40% of cases are idiopathic/familial. Approximately one quarter of these in turn possess a mutation in the bone morphogenetic protein (BMP) signalling pathway. Overall, ~29% of all inherited PAH mutations are substitution nonsense mutations – a very significant percentage. Ataluren rescued, *ex vivo*, a high percentage of BMPR2 and SMAD9 substitution nonsense mutations and also rectified BMP signaling. Functionality was restored in part to these proteins. The proliferative phenotype of PAH endothelial and vascular smooth muscle cells was reduced via Ataluren readthrough. Of practical note was that rescue was produced at doses within the therapeutic range employed in current clinical trials of readthrough for cystic fibrosis.

This demonstrates that readthrough offers considerable practical clinical benefits within the next few years, agreeing further with proof of concept for a diverse range of conditions [1] (Figure 12).

Readthrough is particularly attractive as it targets a mutation-type rather than a specific gene or pathway, such as with imatinib mesylate. Further, readthrough would appear appealing as a simple, safe, drug therapy approach as oppose to other, more cumbersome means, to rescue nonsense mutations [293,294].

The latest generation of readthrough agents, represented by the FDA approved agent, Amlexanox, offers up very good opportunities to apply readthrough as a translational effort in the clinic. Already,

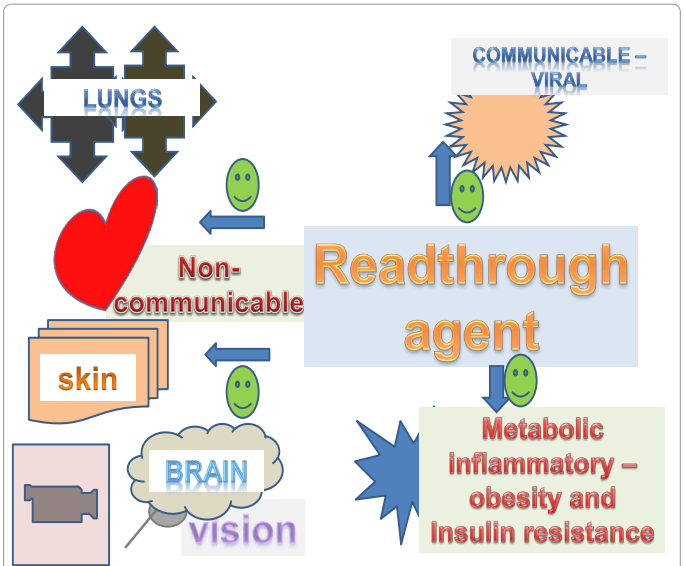

Figure 12: Read through agents have shown 'proof of concept' for numerous non-communicable diseases [1] - also for communicable viral disease [1]. Amlexanox has demonstrated *in vivo* proof of concept for management of inflammatory-related obesity and insulin resistance [180].

Amlexanox is being organized in clinical trials as an inflammatory modulator for management of obesity/diabetes [295] (Amlexanox and innate immunity). The applications for Amlexanox would appear many and varied as suggested in this article and also earlier [1] (Figure 13). Certainly, a point of concern would be the effect of chronic administration of this agent, despite its proven safety margin. I have attempted to analyze this issue by examining the precise relationship of noncanonical NF-kappaB kinases (a target for Amlexanox) in the face of pathogen challenge (viral, bacterial). It would appear that noncanonical kinases, although overlapping in some respects with other NF-kappaB regulatory circuits, have a role to play in themselves, This is borne out with their activity in the presence of various pathogens (Amlexanox and innate immunity). It is not easy to predict at this stage how long term modulation of that signalling axis could affect infection response. Having said this, taming the NF-kappaB axis would appear beneficial in cancer (Cancer, TBK1, Ikkepsilon and Amlexanox), heart and periodontal diseases (Periodontal disease, Ikkepsilon, TBK1 and heart disease) as well as neurological inflammatory disease/ neurodegenerative disease (Neurology – Neuroinflammation) amongst other conditions. Accepting this as the case, then Amlexanox may be viewed as a powerful new therapeutic agent with a multitude of clinical applications.

One point deserves emphasis, and that applies to bioavailability. This has been introduced earlier [1], particularly in regard to whether effective therapeutic levels of Amlexanox may be able to negotiate the blood brain barrier. Given the low solubility of Amlexanox in aqueous solutions, it may be necessary to modify this drug in order to broaden its potential to negotiate a range of targets, for example, within the central nervous system – both as a readthrough agent and as an inflammatory modulator. For example, in order to improve the solubility of Ibuprofen, a propanoic acid and nonsteroidal general anti-inflammatory drug (NSAID), the lysine salt of this drug has been created, enabling its use intravenously [295]. Ketoprofen, a propanoic acid and NSAID, crosses the blood brain barrier [296], and may be given intravenously. Careful examination of this aspect of bioavailability may need to be achieved in animal models of human disease to structure the best means to formulate Amlexanox preparations to reach their various targets. Figure 14 summarizes possible challenges facing Amlexanox therapeutics in the clinic.

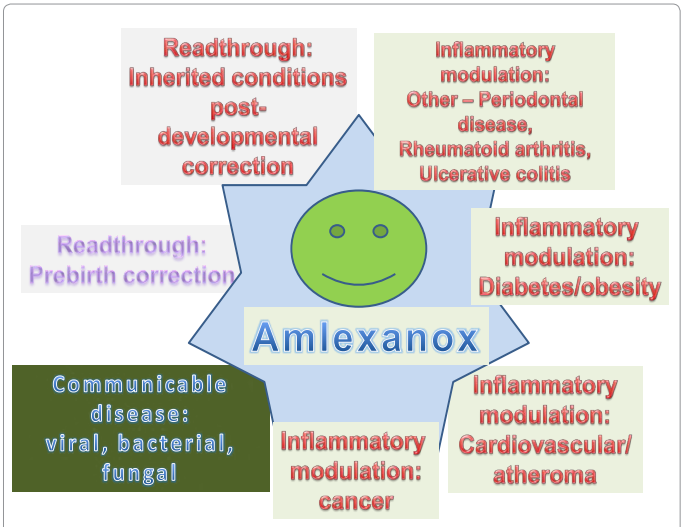

Figure 13: Amlexanox therapeutics has many potential applications from prebirth rescue of nonsense-mediated genetic defects, post-developmental nonsense mutational correction, introduction of resistance to invading pathogens *via* read through, moderation of the infllammatory response and management of cancer, heart disease and other immune-mediated conditions Figure 13 Amlexanox therapeutics has many potential applications from prebirth rescue of nonsense-mediated genetic defects, post-developmental nonsense mutational correction, introduction of resistance to invading pathogens *via* read through, moderation of the infllammatory response and management of cancer, heart disease and other immune-mediated conditions

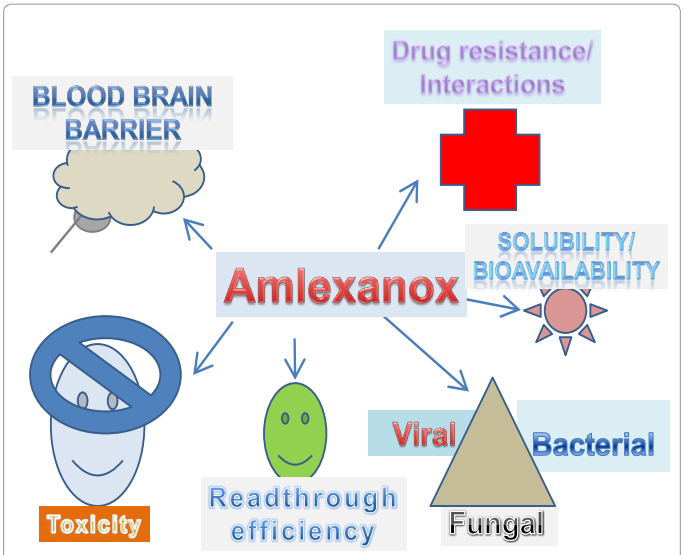

Figure 14: Various points need to be taken into consideration when planning long term Amlexanox use. These include: negotiating the Blood Brain Barrier for effective reach of nervous system targets; toxicity (prebirth & general); drug resistance/drug interaction; bioavailability and effect on pathogen exposure of the host.

Years of experience with Amlexanox in Asia as well as in the USA stand in favour of the safety margin and usefulness of this agent. The old maxim: res ipsa loquitur may well apply here, viz: facts speak for themselves. My proposal for use of strategic tools, such as Amlexanox, in our current 'battle' against communicable and non-communicable diseases Worldwide may be likened to a Sir Winston Churchill quote, the famous British figure of State: 'Give us the tools, and we will finish the job'.

Acknowledgement

I should like to thank my mother for many practical and encouraging discussions throughout the preparation of this article. I also thank the reviewers for their valuable critique of the manuscript.

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