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Dietary indole glucosinolates and skatole: Do they have a role in idiopathic pulmonary fibrosis: Case series data and review

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Abstract
Background: Idiopathic Pulmonary Fibrosis appeared as a new clinical entity alongside fibrotic radiographs of known occupational lung disease from the late 1970's. The veterinary world also recognised an acute fatal lung injury and a slower progressive fibrosis in farm animal's especially older males. They identified the pneumotoxicant to be linked to forage Brassica ingestion and reproduced both conditions in the field by varying exposure levels.
In the UK, dietary Brassica intake has increased by the progressive replacement of sunflower oil by the Brassica derived oilseed rape in foods.
Method: We have examined the effects of ingestion of this oil in 8 healthy medical staff and measured 10 serum mediators relevant to IPF. We have clinically followed a group of patients (9 IPF and 7 Fibrotic-NSIP) who also reduced their dietary rapeseed oil intake by 70%.
Results: The medical staff showed elevations in serum tumour necrosis factor-α (p=0.036), caspase-3 (p=0.0046), matrix metalloprotease-7 (p=0.011), vascular endothelial growth factor (p=0.045), cardiac troponin (p=0.038) and plasma thiocyanate (p=0.028). Alpha-1 antitrypsin levels decreased (p=0.0024) while transforming growth factor β, cytochrome C and Pink1 showed non-significant reductions in levels. With daily oil intake of 20 ml, Caspase-3 and cardiac troponin showed a positive correlation (r=0.8214: p=0.0286), as did thiocyanate, caspase-3 and vascular endothelial growth factor (r=0.8986: p=0.0241)). A negative correlation was for transforming growth factor β and VEGF levels (r=0.8214: p=0.0286) and cytochrome C and thiocyanate (r=0.9820; p=0.0021). For the patients, coughing resolved within 4 weeks with reasonable stability of forced vital capacity, walking tests and HRCT scores unless impacted by COVID-19 infections or other significant medical events. The mean patient follow-up was 4.8-5.2 years.
Conclusion: We discuss the findings within these 2 groups and review the veterinary data and information on the Brassica family and the effects of their released glucosinolates.

Keywords: IPF, Diet, indole-Glucosinolates, 3 Methyl Indole (Skatole), lung Fibrosis in livestock, TNFα, MMP-7, Caspase-3, VEGF, Canola oil

Introduction
Idiopathic pulmonary fibrosis (IPF) is increasing around the world and is now the commonest interstitial lung disease. The UK office of National Statistics showed that age standardised mortality rates rose from 1.66 per 100,000 in 1979, to 7.6 per 100,000 in 2022. The International Classification of Disease codes (ICD) did change at the millennium from ICD-9 (Post-inflammatory fibrosis; 516.3) to ICD-10 (IPF: 84.1); with diagnostic accuracy considered high for IPF-clinical syndrome (formerly cryptogenic fibrosing alveolitis) suggesting a true increase [1-4].
In the late 1970's, IPF appeared as a new clinical entity previously unrecognised alongside fibrotic chest radiographs from coal miners, silicon and asbestos workers [5]. The industrial areas of England and Wales had more cases especially in men with epidemiological studies suggesting metal work and wood burning to be a risk. The possibility of prescription drugs was questioned, as several including amiodarone and nitrofurantoin already had known fibrotic effects [5-7]. The striking link with older age led to suggestions of a degenerative process in the lung, although HLA-links were absent and familial cases a minority.

Data showed a higher risk for current (hazard ratio 1.66) and former smokers (HR 1.42) relative to never smokers [8-10].

The condition presents with cough and breathlessness with fibrotic changes in the lower lobes in a sub-pleural distribution on CT scans. A greater incidence of lung cancer occurs along with increased gastro-oesophageal reflux and type-2 diabetes. Under-activity of the thyroid is common with arterial disease greater than that seen for emphysema. Pulmonary hypertension arises in 10-32% of cases as the disease progresses with asymptomatic hepatic fibrosis described in one-third of patients along with serum auto-antibodies and telomere shortening. The causal agent for IPF remains unknown but clearly needs to unify all these features [9-12].

In this regard, past scientific and veterinary data warrants consideration as it may link many of the above features to the ingestion of glucosinolates from the Brassica family of vegetables especially indole glucosinolates derived from L-tryptophan.

A report in 1944 by a Canadian vet, described the 'poisoning' of cattle following an autumn change of pasture to tall lush grass with Forage Brassica containing Rapeseed crop [13]. This produced an acute interstitial pneumonia (AIP) with death from respiratory failure within days, especially noted in healthy but older cattle that overgrazed. The condition

was referred to as 'fog fever', with the Canadian's noting that rapeseed leaves displaying a purple colour and early frosts produced more cases compared to other years. Similar reports occurred during the 1970's in Europe, the US and UK leading to a major study and thesis by Roger Breeze (Glasgow Veterinary College in 1973) to determine the cause [14]. His extensive work showed that the amino acid L-tryptophan (L-trp) present in lush grass and forage Brassica (rape, kale, turnip and swede) was metabolized within the bovine rumen by the bacterial microflora to a highly lipophilic compound called 3-methyl indole otherwise known as Skatole. Oral daily doses of L-trp (0.4gm/kg) or skatole (0.1gm/kg) given to cattle reproduced acute alveolar damage, with a 23% conversion rate of ruminal L-trp to skatole [15]. The D-isomer of tryptophan did not undergo this metabolism [16]. Carbon-14 labeling showed skatole to be rapidly cleared from the blood and selectively concentrated within the alveolar type I & II cells and lung Clara cells as the only site of injury seen in the cattle. Apoptosis of the alveolar cells disrupted the alveolar surface with fluid leakage into the airspaces that risked death from respiratory failure, and this was the basis of 'fog fever' [17-19]. These animals also developed acute 'emphysema' from barotrauma, a known feature of bovine respiratory difficulties but unrelated to the human condition.

Studies of the alveolar cell's P450 enzyme system within the endoplasmic reticulum, showed skatole to be metabolized to a pneumotoxicant called: 3-methylene indolenine (or 3MEIN) through dehydrogenation. 3MEIN had lethal effects upon alveolar cells within 6-24hrs through covalent binding to proteins and nucleic acid, producing protein adducts with DNA fragmentation and apoptosis [20]. Phase-2 enzymes of the glutathione system could reduce intracellular 3MEIN levels through direct covalent binding if replete. Skatole induced AIP produced alveolar type-2 cell proliferation in surviving animals, while further attacks of 'Fog Fever' produced a chronic slowly progressive respiratory impairment over many months associated with diffuse alveolar fibrosis [15, 21-25]. This condition was called cryptogenic pulmonary fibrosis and was well described in cattle, goats, sheep and horse but not in pigs. The histology showed chronic alveolar exudates with fibrotic tissue infiltrated by neutrophils, macrophages and fibroblasts resembling the condition in man and suggesting a good animal model [15, 16]. Diffuse alveolar fibrosis was successfully reproduced in goats, steers and dairy cattle by once-weekly low doses of oral L-Trp (0.4 gm/kg**)** or skatole (0.1 gm/kg) given over several months**.** This avoided death from AIP but produced a slow fibrosing alveolitis associated with raised blood levels of 3MEIN relative to control animals [15, 26]. Oral antibiotics were protective, through inhibition of the microfloral conversion of L-trp to skatole. Phenobarbitone, an inducer of the P450 enzymes system, increased skatole's toxicity while the CYP-inhibitor piperonyl butoxide reduced it. In man, low levels of skatole are present within the healthy colon (0-5 µg/g) but cigarettes contain high concentrations at 1.4 µg per cigarette and smoking already has strong but unexplained links to IPF [15, 27].

The lung CYP2F1 enzyme is a member of the P450 cytochrome system, and was shown to have a unique 'active site' that drove all skatole via dehydrogenation to the single toxic metabolite of 3MEIN; with cell concentrations as low as 0.1µm able to induce DNA damage and apoptosis [27-31].

Human lung tissue from transplants confirmed high concentrations of the 2F1 enzymes within the epithelial cells that could produce 3MEIN [32]. The 2F1 gene is selectively expressed in the lung and well conserved in mammals with 80-85% homology. The Goat CYP 2F3 enzyme shows high conversion rates to 3MEIN with 82% homology to human 2F1 [32]. Other human lung CYP enzymes include (CYP-1A1, -3A5, -1B1, -2B6, -4B1, -2E1, and 2F1); of these only CYP1A1, CYP2E1 and CYP2F1 can metabolize skatole to 3MEIN. CYP1A1 and CYP2E1 preferentially metabolize skatole via hydroxylation, epoxidation and particularly oxidation, thereby reducing the dehydrogenation product of 3MEIN [33, 34]. Differing CYP enzymes in pigs produce different metabolites explaining their resistance to skatole induced lung injury [35].

Seven allelic variants of 2F1 are reported in Caucasians, but little additional information is available and bio-activators unknown apart from 3MEIN metabolites [36-40]. Alveolar type-2 cells contain the highest levels of P450 enzymes relative to type-1 cells, which lack organelles leaving them more vulnerable to cell injury. The testicular hormone androstenone has shown inhibitory effects upon the CYP2E1 promoter region, and is considered to influence its metabolic activity and may explain the male animal predominance seen in 'fog fever' [35].

With the veterinary data linking both acute and slow fibrotic alveolar injury to the ingestion of brassica plants, this raises the question as to whether such observations have been previously described in man. Certainly the Spanish Toxic Oil Syndrome (1981) is one example that arose suddenly in a town north of Madrid. Here, over 20,000 people were acutely affected by a severe respiratory problem thought to be a virulent adenovirus. Early symptoms included cough and breathlessness with pulmonary infiltrates and adult respiratory distress syndrome that led to >800 deaths [41-43]. After 6 weeks without an infectious agent identified, the cause was linked to a shipment of industrial grade rapeseed oil, bottled at the port and sold door to door by street vendor

as olive oil. This oil was presumed to contain a toxin such as aniline or pesticides, but extensive analysis of the recovered oil and *in-vivo* animal studies did not reproduce its toxicity nor confirm aniline as the definite causal agent. This led several experts to suggest that the triglyceride fat "Erucic Acid" within the rapeseed oil itself was responsible for the disaster [41, 44].

Despite brief exposures to this oil (2-8 weeks), further symptoms followed the respiratory component including severe myalgia, hepatitis, systemic inflammation, pulmonary hypertension and arterial disease [45]. 20% of patients with initial radiological resolution of their AIP later showed fibrotic change [46]. Later still, a condition resembling scleroderma arose in 21% of survivors, with chronic pulmonary hypertension in 8%, chronic liver impairment in 3%, hypothyroidism in 7% and diabetes 8%, all of which was unexplained [47]. Pulmonary artery histology showed muscular hypertrophy, plexiform lesions and arterial lumen narrowing similar to the pulmonary hypertension in IPF. Despite a clinical picture of inflammation and autoimmunity, no auto-antibodies were consistently identified in the patients. In 1994, UK vets reported a new illness in cats and dogs fed tinned pet food supplemented with vegetable fat from rapeseed-meal. These pets developed a scleroderma-like illness with thickened inflamed skin, fur loss, wasting and systemic inflammation similar to the toxic oil syndrome. The vets also reported multiple malignancies in a percentage of the animals that died. Recovery was very slow following a dietary change to dried food free of rapeseed products [25]. Rapeseed meal is a protein and amino acid rich product used in animal feed following oil extraction from the seeds.

L-trp itself has also been linked to the Eosinophilic Myalgic Syndrome after Health Shop supplements (1.6-4gm/day) produced lung infiltrates in some patients. A clinical pulmonary hypertension was described along with muscle pains and eosinophilia. The manufacturing method in Japan was considered causal, despite individual reported cases solely attributed to high intakes of L-trp via the diet [49, 50].

The Brassica family includes broccoli, cauliflower, cabbage, radish, kale, brussel sprouts, turnip, swede and the oilseed plants of mustard and rapeseed [51-53]. All contain glucosinolates (GL) which are Sulphur based compounds offering anti-microbial, anti-fungal and helminth protection for the plant and are limited to the Brassica family with the exception of Indian cress, capers and papaya. The predominant amino acids that constitutes the R-side chain classifies the GL class:- methionine, alanine or leucine (Aliphatic-GL), phenylalanine or tyrosine (Aromatic-GL) and L-tryptophan (Indolic -GL's) see figure 1.

GL's make up 1% of the plant's dry weight and 5% of their seeds, giving a leaf content of 14-25 µmol/gm and oil seeds content of 55 µmol/gm with higher levels within root systems for protection [54].

Intact GL's within brassica are stable and non-toxic in contrast to their breakdown products that include >130 different metabolites generated by GL hydrolysis; with many so reactive that assays and *in vivo* effects are unknown [52, 55-58].

Ingested Brassica can release their intact GL's during chewing and chopping with absorption from the stomach or breakdown by gastric acid, gut bacteria or by its own plant enzyme 'myrosinase'. Active myrosinase within Brassica can hydrolyze GL's unless it is already inactivated by cooking, processing or storage (Figure 1). The absorption of intact GL's is greater from brassica juices or liquids as they are no longer encased in plant tissue. Myrosinase-like activity also exists within the microflora [59, 60].

Gut absorption of GL metabolites is excellent with many metabolized by the liver to conjugates of glutathione with renal excretion as N-acetyl cysteine [61, 62]. Radioactivity data shows a biliary enterohepatic circulation of many metabolites with high radioactive uptake by the intestinal mucosa, liver, lungs, kidney, bladder and spleen [52].

Figure 1: The GL hydrolysis products fall into 4 main groups: stable and unstable isothiocyanates (ITC), thiocyanates (SCN) and nitriles. The stable ITC have anti-cancer properties especially sulforaphane and allyl-ITC [56, 63-68]. The unstable ITC are all indoles derived from L-trp, whose side-chain may undergo rapid transformation including a metabolic pathway to skatole production (Figure 2). Concerns over mutagenicity have been raised for some indole-ITC metabolites following observations in cell systems [69-72]. The thiocyanates (SCN) have effects upon the mitochondrial electron transport chain while the nitriles produce unstable breakdown products with adverse liver, kidney, thyroid and nervous system effects described in animals [56].

Most Brassica vegetables consumed within our diet are cooked, reducing their total GL content by >60% and inactivating myrosinase. By contrast, farm animals ingest unprocessed forage brassica with its high GL content along with additional rapeseed meal supplements [73, 74].

Rapeseed oil (RSO) and meal has attracted significant attention due to cardiac injuries in animals related to erucic acid and adverse GL effects [44, 75-77]. This resulted in a European and North American law (1996) limiting rapeseed cultivars to an erucic acid content < 2% and GL content <30 µmol/gm; Called 'double-low' canola [57, 78, 79]. In India, China and eastern countries this policy has not been adopted. The 'double low' has mainly reduced the toxic aliphatic GL in RSO without reducing the indole-GL content. Past attempts to removal of GL from RSO has required sulphuric acid extraction leaving the oil rancid and unusable. Geographical regions and fertilizers can increase GL content up to 10-fold, while UV light reduces GL levels within exposed leaves [80, 81].

The commonest Indole-GL in RSO/canola is Glucobrassicin (chemical names are 3-indolyl methyl GL or indol-3-ylmethyl GL) [82]. This Indole has a metabolic pathway to skatole figure 2; commencing with rapid non-enzymatic autolysis to a nitrile (Indole-3-acetonitrile) at gastric acid pH <4.0, followed by metabolism to Indole-3-acetamide and then Indole acetic acid (IAA) and finally to skatole (3-methyl Indole) [82, 83] Nitriles are produced in acid conditions and are well absorbed from the stomach and upper GI tract in man [83-86]. Skatole has not been extensively studied in man, although intestinal levels are high in gut dysbiosis (80-100 µg/g**)** [87, 88]. The biosynthesis of IAA (The precursor of skatole), is increased by flavonoids abundant in RSO and diets rich in animal proteins which increase gut IAA levels 3-fold. Raised intestinal sugar concentrations increase the decarboxylation of IAA to skatole [55, 87, 89].

In the UK, the use of sunflower oil began to decline in the late 1970's with imports of RSO rising from 6,000 metric tonnes /yr in 1970 to >724,000 M.T. in 2023 (Published Feb 2024 Agriculture + farming UK). The oils of sunflower, soya, palm and RSO do not contain free amino acids and L-

trp is undetectable (https://www.feedtables.com/charts/amino-acids/oils), although costly extra virgin olive oil is not infrequently mixed with lower cost oils like canola or palm for economic gain [90]. The total content of GL's is highest in cold-pressed RSO/canola oil at 11.9 µmol/gm, relative to solvent extracted oil (6.02 µmol/gm) with the indole-GL of Glucobrassicin constituting 20.1% and 15.2% of that total GL content respectively [91-93]. From these observations and additional data yet to be discussed, could there be any plausible link between increased indole GL exposure and IPF that warrants investigation?

We present data from 8 healthy medical staff following a RSO-free diet followed by regular RSO intakes with measurement of 10 mediators relevant in IPF. In addition, we clinically followed a small series of untreated patients who reduced their RSO intake by 70% with forced vital capacity (FVC), shuttle walk tests (SWT) and High Resolution Chest CT follow ups as indicated.

All patients gave written informed consent for publication and the most of the medical staffs are co-authors.

Methods

The patients

Nine UIP/IPF patients and 7 F-NSIP (Fibrotic-nonspecific interstitial pneumonia) were clinically followed prospectively for 2-8 yrs. None of the UIP patient received anti-fibrotic drugs initially as they either did not fulfill the treatment criteria or refused referral. For F-NSIP, anti-fibrotics were approved in Nov 2021 and available by spring 2022 in the UK if treatment criteria were fulfilled [94]. Our patients were originally diagnosed by multidisciplinary review of clinical history and examination, occupation, radiology and lung function in line with the Official ATS/ERS/JRS/ALAT guidelines of 2011 and later 2018 [2, 95]. No patients had a lung biopsy.

Patients reduced their dietary intake of RSO by close to 70%**.** This reduction was not individually recorded, but patients substituted common food products listing RSO or vegetable oil for those using sunflower or olive oils while shopping. They were aware that restaurant and take-away food and bread products usually contained RSO. Standard 6-monthly outpatient follow up continued with measures of oxygen saturations, FVC and shuttle walking test with HRCT scans if clinically indicated. Like all patients with lung fibrosis, they were instructed to make contact if their clinical status changed between visits requiring re-assessment. Restrictions during the COVID-19 lockdown (2020+2021) did affect some assessments in patients concerned over COVID-19 exposure at the hospital. Some FVC values fell during lockdown but recovered once normal activity levels were resumed.

The HRCT chest scans were scored by 2 consultant chest radiologist (AW & RC) according to the method of Hansell & Wells [96]. This scoring assessed 4 levels in the lung (Aortic arch, carina, inferior pulmonary veins and lung bases), with assessment for ground glass change, septal lines, honeycomb and emphysema. The scoring system for each abnormality was: (Score 0: no change, score 1: <5% change, score 2: 5-25%, score 3: 25-50%, Score 4: 50-75% and score 5: >75% change). This gave a total possible score of 160 for both lungs with increases in scores indicating progressive changes. The chest radiologist's did not have lung function data but were aware of any intercurrent infections such as COVID-19.

For UIP/IPF patients, septal lines and honeycomb change were present and affected the lung bases without any significant ground glass change; while F-NSIP patients showed basal septal lines (Patient B, C, D) or basal and lower lobe septal lines (Patients A, E, F, G) without honeycomb change. Ground glass change was seen in the affected areas along with some scattered patchy ground glass change seen in unaffected areas.

For each patient, the first FVC measurement (Pre-dietary change) is shown within a green box in the tables. The mean change in FVC per year was then calculated by subtracting the most recent FVC reading from the FVC value once dietary change had begun. This difference was then divided by the observation period in years to produce a positive or negative value for FVC change in ml/year. A similar approach was used for SWT. The total HRCT scores over the observation period are recorded within brackets in the tables.

Consent: All patient gave written informed consent for inclusion in this case series.

Medical staff taking RSO

Day 1-14: Eight healthy medical staff (nonsmokers: mean age 55yrs, range 45-64 yrs; 4M:4F) undertook a strict rapeseed oil free diet for 14 days involving the checking labels on all foods and dressings with avoidance of restaurant and take away food. They ate home prepared dishes, avoided fats and oils containing RSO or listing vegetable oil along bread products.

On Day 15: Baseline blood samples were taken for measurement of 10 mediators relevant to IPF. RSO was then commenced at 10ml/day (double-low cold-pressed canola oil) for 4 weeks. This was generally added to their evening meal while other sources of RSO were still avoided.

Week 2+4 further blood tests were taken while dosing RSO 10ml/day.

Day 1-14 A second baseline RSO-free diet was commenced.

Day 15- After a second RSO-free baseline blood sample, RSO 20ml/day was commenced with a final week 4 blood sample upon completion.

The corresponding author continued RSO at 30ml/day and then 40ml/day each for 2 weeks with a blood samples upon dose change to assess for any dose effect once samples were analyzed.

Baseline blood sample analysis

Serum and EDTA/plasma samples were collected and aliquoted into multiple small tubes with storage at -80^{oc} until analysis. Plasma thiocyanate levels (SCN) were sent to the Health and Safety Reference Laboratory, Buxton, Derbyshire, UK. In house measurement of cytochrome C, Pink-1, caspase-3, transforming growth factor β-1, tumor necrosis factor-α, Matrix MetalloProtease-7, Vascular Endothelial Growth Factor were performed using purchased ELISA kits. Each mediator was assayed as one experiment on the day with close adherence to kit instructions. Cardiac Troponin-I was sent fresh to our laboratory along with serum for alpha-1 antitrypsin assay. The details of the ELISA kits used and method are listed after the conclusion.

Statistical analysis

Results were analyzed by column statistics for group means with 95% confidence intervals (CI) using the statistical package of Prism 3 and SPSS. The effects of RSO avoidance and RSO intake upon mediator levels were analyzed by Paired t-test with significance taken at $p<0.05$.

Spearman's rank-order tests for correlation coefficients were performed for the 20ml RSO dosing where significant changes in 6 mediators were noted, in order to determine the strength and direction of the monotonic relationships between two variables when normality was assumed.

Results

The Patient: The demographics of both groups are shown in table 1. Most patients were in their 70's with a male predominance for the F-NSIP group. Nine UIP/IPF patients were followed for a mean of 4.8 years, and seven F-NSIP patients for a mean of 5.2 years following the dietary exclusion. Three patients had auto-antibody without evidence of clinical disease. The annual results for all patients are shown in the tables with any change in clinical status or treatment indicated. Dramatic resolution of cough was reported by most patients within 4 weeks that served to increase dietary compliance.

UIP/IPF (Patients 1-9)

Table 2: shows the first recorded FVC value (within a green box), prior to dietary change. That initial FVC ranged between 59-115% for the group. Following dietary change, FVC values showed reasonable stability for the 9 patients in the absence of other medical events with 4 subjects showing improvement in their FVC. Overall the change in absolute FVC value (mls/year) varied between +160 mls to -170 mls/year for the group with a relative change of +14% to -13%. Most FVC reductions occurred due to COVID-19 infection (3 patients) after lockdown ended or followed significant medical events (4 patients) as indicated on the tables**.** The expected annual decline in FVC for untreated IPF is 130-210 mls per year. This value was reached in patient 5 (vertebral fractures) and patient 9 (post COVID-19 vaccine exacerbation).

Table 3: Shows the SWT change during observation to range between +23 m to -45 meters/year, with patient 4 excused after 2019 due to anaemia.

Total HRCT scores increased in 4 patients, due to increased septal lines (patient 1+6), honeycomb change (patient 5), and for patient 9, ground glass change in 2019 followed by septal lines and honeycomb change post Covid vaccine in 2021/22. The remaining 5 patients were stable.

During the observation period 3 UIP patients sadly died: Patient 4: from transforming myelodysplasia with stable respiratory symptoms. This patient had also suffered severe daily abdominal pains for 10yrs without explanation which completely resolved on RSO-reduction leading to its complete avoidance by the patient. Patient 8: died from complications of a fall without new chest x ray changes. Patient 9: died during sleep at home, with valvular heart disease, diabetes and cardiac and pulmonary fibrosis listed at post mortem. This patient had deteriorated with each of 3 COVID-19 vaccinations in 2021 resulting in an oxygen requirement. Tertiary referral was eventually agreed to by the patient, but anti-fibrotic drugs were not commenced due to a life-long aversion to prescription drugs. Patient 5: developed multiple osteoporotic fractures (L1-L4) affecting SWT and FVC measurements. Salmon calcitonin was used for pain control with later balloon kyphoplasty to the affected vertebrae. Patient 6: developed effort chest pain requiring a coronary artery stent. Cardiac rehabilitation led to a COVID-19 infection and anti-fibrotic referral in 2023. Patient 7: required a permanent pacemaker with post insertion pacemaker infection requiring inpatient antibiotics with COVID-19 contracted as an inpatient with a good recovery.

F-NSIP (patients A-G)

Table 4: the initial FVC (in green box) for the group varied between 47-106% predicted. Following dietary change, FVC values showed reasonable stability for the patients in the absence of medical events. Overall the annual FVC change varied between +25 to -400mls/year for the group (relative FVC change being +1.16% to -10.4% per year). Most declines in FVC were due to COVID-19 infection [97].

Patients A, D, F and G contracted COVID-19 in 2022. Patients A+D recovered without FVC or SWT changes and were observed. Patient F showed a 6% fall leading to anti-fibrotics referral. Patient G had a 19% FVC fall post-Covid with anti-fibrotic referral. Here bronchoscopy was declined and an 8 weeks steroid trial substituted with an additional 30% FVC decline and an oxygen requirement (4L/min) that did not change following nintedanib. Patient E: died after an upper respiratory tract infection complicated by pneumonia 7days post-hospital discharge.

Table 5: shows the SWT changes to vary between +36 to -34metres/year with most reductions following COVID-19. Patient E did not perform SWT due to osteoarthritis. HRCT scores increased in all patients by 1-32 points, indicating progression with marked increases following COVID-19 infections. Patients (A, C, D, E, F) showed increased ground glass and septal lines change while patients (B+G) showed ground glass change, septal lines and new basal honeycomb change post Covid infection.

The medical staff results

Seven out of 10 mediators showed significant changes with RSO dosing and data presented in Table 6+7 and Figure 3 +4 Plasma thiocyanate (SCN) levels increased significantly from the 1st RSO-free baseline mean of 50 µmol/l to 73 µmol/l (p=0.0283) following 10ml/day. The 2nd RSO-free baseline was similar at 57 µmol/l and increased to 76 µmol/l **(**p=0.0286) following 20ml/day (table 6). Interestingly, 50% of the medical staff developed a significant dry cough during 20mls dosing that resolved two weeks after its cessation. SCN does produce cough and has a plasma t½ of 14 days that may explain this. SCN's are products of GL hydrolysis, figure 1. SCN also showed a positive correlation with VEGF and caspase-3 levels (p=0.0241: r=0.8986) and a negative correlation with cytochrome C at 20 ml/day (p=0.0021: r=0.9820). Cytochrome C forms step 3 of the mitochondrial electron transport chain where SCN can have inhibitory effects.

In non-smokers, expected SCN levels are 33-38 µmol/l, while levels of 59-70 µmol/l are seen with smoking 5-9 cigarettes/day and 87-99 µmol/l with >25 cigarettes/day [98, 99]. Plasma SCN levels >60 µmol/l generally distinguish smokers from non-smokers and high Brassica intake may increase levels by 10 µmol/l [99]. In smokers, hydrogen cyanide from tobacco is the source of SCN, with reports suggesting true SCN blood levels are 10-fold greater than

plasma levels through additional binding to albumin and red blood cells.

Serum Cardiac Troponin-I levels rose from the 1st baseline of 1.04ng/l (95% CI 0.01-2.0) to 1.9ng/l (CI 0.4, 3.3: p=ns) on 10mls/d. The 2nd baseline mean was =1.13(0.2, 1.9), which increased to 2.35 ng/l (CI 0.76, 3.9: p=0.038) following 20ml/day. Troponin levels remained within the normal range at all times but showed positive correlation with caspase-3 (p=0.0286: r=0.8214). RSO doses of 30-40mls showed a greater change (Table 7).

Tumour Necrosis factor-α (TNF α) normal range 5-27 pg/ml

The medical staff had normal serum TNF levels of 15.4-16.6 pg/ml for both RS0-free baselines. These levels increased to means of 28.8 + 36.0 pg/ml following 10+20 ml/day dosing with significance for 20 ml/d: P= 0.036 (table 6). TNFα is released by macrophages, fibroblasts and epithelial cells upon activation and also tissue injury and has a t½ of 4.6 minutes in the circulation.

Caspase-3 levels; normal range <200 pg/ml and half-life is 8-11 hours.

The 1st RSO-free mean was 155 pg/ml, following which levels increased continuously with a 2nd RSO-free mean of 325pg/ml. After 20ml dosing this value was 466 pg/ml (p=0.0046). A 3-fold increase occurred for the subject who continued dosing to 30+40mls/day resulting in a serum level of 958 pg/ml. Caspase-3 indicates activation of apoptosis and was shown to have a positive correlation with cardiac troponin during the 20ml dosing (p=0.0286, correlation coefficient r=0.8214). This may reflect the effects of TNF upon both mediators. There was also a strong positive correlation between thiocyanate, caspase-3 and VEGF (p=0.0241; r=0.8986) at 20 ml, again suggesting TNF effects: Figure 4.

Vascular Endothelial Growth Factor (VEGF) t½ 30minutes.

Baseline RSO-free levels were 38 pg/ml (normal serum <130 pg/ml) and rose to 201 pg/ml (p=ns) following 10ml/d. The 2nd RSO-free baseline was 164 pg/ml and rose significantly to 406 pg/ml (p=0.045) after 20 mls/day. This glycoprotein is elevated in IPF with effects angiogenesis with secretion mainly by alveolar epithelial cells. VEGF showed an inverse negative correlation with TGFβ1 levels (p=0.028: r=0.8571) after 20mls/day and TGFβ1 can exert inhibitory effects on VEGF. A positive correlation occurred for VEGF, SCN and caspase-3 (p=0.0241; correlation coefficient r=0.8986) [100].

Matrix Metalloprotease-7(MMP-7) normal range 1.07-4.40 n g/ml

Baseline levels were 1.72 ng/ml and rose to 9.8 ng/ml after10mls p=0.0116. The 2nd baseline was 2.78 ng/ml and rose to 5.9 on 20 ml/d p=ns; however 2 samples unexpectedly failed this assay affecting the mean value and 20 ml analysis [101-103].

Alpha-1 antitrypsin (A1AT), normal range 1.1-2.1gm/L.

All the medical staff had normal baseline levels that were unchanged by RSO 10ml dosing. During 20 ml dosing, A1AT levels showed a significant decrease to 1.15 gm/l (p=0.0024) that may indicate uptake by endothelial cells (table 6). The t½ of this plasma protein is 4-5days.

Transforming Growth Factor-beta-1(TGF-β1), (normal range of 2000-12000 pg/ml).The mean RSO-free baselines were 6356-6362 pg/ml. TGF-β1 levels showed a unexplained 9% fall after 10ml + 20 mls (– 599 pg/ml, - 608 pg/ml respectively) and had a significant negative correlation with VEGF levels (p=0.0286: r=0.8571) at 20 ml dosing. TGF-β1 drives fibroblast proliferation and matrix deposition. Its t½ is 3 minutes.

Pink-1(PTEN-induced putative Kinase-1); t½ of 27 minutes.

Baseline mean serum levels were high at 1743 pg/ml with the normal range of 320-700 pg/ml.These levels decreased by 6-15% with RSO dosing of 10+20 mls/day (-111 pg/ml and -353 pg/ml respectively, p=ns). Pink-1 protects the mitochondria from oxidative stress and serum levels are not reported to be higher in youth but do decline with biological age increasing mitochondrial vulnerability to oxidative stress [104-106].

Cytochrome C: Our data showed normal serum levels (<212 pg/ml) that showed a 9% decrease on 20 ml dosing producing a significant negative correlation with thiocyanate levels (p=0.0021: r=0.9820). Cytochrome C is involved in mitochondrial injury and activation of the intrinsic apoptotic pathway where thiocyanates have effects upon the mitochondrial electron transport chain. t½ is 5-6 days [107].

Discussion

Our 16 patient's constituted a very small group with a mean follow up of 4.8-5.2 years on a RSO-reduced diet. For the 9 UIP patients, FVC remained reasonably stable and improved in 4 subjects. The HRCT scores showed no change in 5 patients, but increased slowly in 3 patients with the 4th impacted by the COVID-19 vaccine.

For the F-NSIP patients, 3 patients showed FVC improvement of +15 to +25 mls/year while other FVC reductions were due to COVID-19 infection. Total HRCT scores increased particularly with infection. COVID-19 was the greatest cause of clinical decline in our 16 patients and this is now confirmed by published studies with adverse vaccine effects also described in 1-3% of ILD patients [108-110]. The clinical course of UIP and F-NSIP varies considerable with a requirement for larger numbers to ascertain stability of the FVC values and HRCT scores if infection is avoided.

The results from the medical staff

Elevations in SCN occur through GL hydrolysis, and represented a smoking rate of 10-15 cigarettes/day for the medical staff. Plasma and urinary excretion of SCN is known to predict lung cancer risk with primary lung cancers arising in 22% of IPF patients; being 5-fold above the general population [8, 111-115]. Electroplating, metal refining and steel production are sources of SCN, with these occupations already linked to IPF development [6].

Reports from China show an association between RSO use in stir-fry cooking and lung cancer development in young non-smoking women; here the risk relates to both the duration and frequency of exposure and suggests an inhaled oxidised product may be mutagenic [116-119]. Studies in rodents exposed to electrically heated RSO vapours within an exposure chamber, also developed lung adenocarcinoma's in 17-25% of animals at 6 months; following a total of 150 exposures each of 30minutes over that period [120, 121]. Brassica vegetables are known for their anti-cancer effects but some GL metabolites can have mutagenic effects which includes possible links of skatole to lung cancer [55, 56, 71]. The 2 nitriles in particular, (Epithionitriles and nitrile) may react with amides in the diet

to produce N-nitroso compounds linked to tumours in over 40 animal species [122].

SCN is a validated predictors of coronary artery disease which affects 28.6% of IPF patients (odds ratio 2.18) compared to 9.8% with emphysema; despite smoking rates of 31% in IPF relative to 98% [4, 12]. Our decision to measure cardiac troponin-I, followed reports of myocardial injury in livestock fed rapeseed meal and the advanced coronary artery atheroma following the toxic oil syndrome, including young non-smoking patients who died from myocardial infarction [44, 55, 77].

Our troponin (cTnl) levels showed a small rise, but published data does not link SCN to elevated cTnl levels, with smokers consistently showing lower cTnl levels relative to non-smoker or ex-smokers [52, 57, 111, 123-125]. Troponin levels are higher in IPF especially when pulmonary hypertension is present but studies are few [126].

Our TNFα levels increased with RSO, and unlike SCN this cytokine can release cTnl from cardiac cells through permeability changes that can be blocked by anti-TNF [52, 57, 111]. TNFα is causally linked to cardiac fibrosis and atherosclerosis with blockade of caspase-3 and matrix metalloprotease shown to prevent these TNFα effects in animal studies. Our caspase-3 levels showed a positive correlation with troponin in the medical staff [127-133]. Macrophages show a dose dependant release of TNFα with cigarette smoke exposure and serum levels are raised in IPF (mean quoted values of 22.4 pg/ml**)** [129, 131, 134-139]. Caspase-3 is an apoptotic mediator activated through TNFα stimulation of the extrinsic apoptotic pathway via surface Fas-Fas-ligand receptor proteins. Inactive caspase-3 is present in most tissues, but surface Fas-Fas Ligands are generally restricted to T cells, liver, lung, heart and gonads. Their activation can destroy a cell in hours through the release of the executioner caspases of 3 and 7 via caspase-8 [140,141]. Fas receptors are upregulated in IPF lung tissue with positive staining for caspase-3 seen in alveolar cells and lavage fluid. Cigarette smoke can also produce a dose-dependent release of caspase-3 [140,142-144].

The VEGF levels (VEGF-A165a + VEGF-A165b) increased significantly in the medical staff. VEGF has 6 isoforms that are seldom separately assayed and may have opposing actions. Lung VEGF levels are 500 times plasma values with high expression on alveolar epithelial cells, and lower expression on vascular smooth muscle, macrophages and fibroblasts. It has a role in the regulation of angiogenesis (New blood vessel formation) at sites of cell injury where it promotes cell survival through restoration of blood supply [145, 146]. In mouse models, over expression of VEGF-A165b inhibits pulmonary fibrosis while VEGF-A165a is fibrogenic, and this may account for some conflicting results from prior lavage data in IPF [145, 147-150].

Lung endothelial cells can secrete a soluble VEGF receptor-1 protein (sVEGF receptor-1) which binds to extracellular VEGF reducing its activation and cellular uptake [146, 149]. Secreted MMP-7 can degrade this sVEGF receptor-1 and increase active VEGF within the microcirculation, allowing uptake by injured endothelial cells and stimulation of their intracellular VEGF-2 receptors to promote angiogenesis; a process that TGFβ can inhibit. Our VEGF data showed a significant negative correlation with TGFβ levels [151]. VEGF can enhance the production of matrix metalloproteases when stimulated by TNFα and may also exert some inhibitory effects upon caspase-3 activation; although our data showed a strong positive correlation between VEGF, caspase-3 and thiocyanate(r=0.8986) [149, 152].

Matrix metalloprotease-7 (MMP-7) increased with RSO dosing to levels seen in interstitial lung disease. This was probably stimulated by TNFα with MMP-7 also involved in atherogenesis, matrix degradation with plaque rupture. MMP-7 is increased by smoking with levels >1.96 ng/ml predicting future cardiovascular events [153-155].

In vitro studies show TNFα to augment MMP-7 expression in macrophages and vascular smooth muscle cells, with pro-MMP-7 also activated by arterial wall oxidants. MMP-7 can cleave pro-TNF within macrophages to its active form along with the release TNFα from epithelial cells to drive an intense inflammatory loop [156-158]. Bleomycin induced lung fibrosis is prevented in MMP-7 knock-out mice where the extracellular release of TGFβ is also eliminated [155, 159]. Plasma MMP-7 and MMP-1 are both raised in IPF with values of MMP-7 >2.6ng/ml +MMP-1 > 8.9ng/m highly predictive of the condition [160-162].

A1AT levels unexpectedly fell during 20ml dosing, changes in secretion are possible, but serum reductions from increased protease binding are not reported [163]. A1AT's is produced by the liver but also released by granulocytes and lung epithelial cells into the local circulation. TNFα can enhance this secretion and A1AT can be internalized by endothelial cells to inhibit apoptosis through direct inhibitory binding to caspase-3. Since caspase-3 levels did rise, this may have provided a signal for A1AT uptake explaining the fall, although the exact details of this uptake process are sketchy. A1AT can reduce TNF-gene activation within endothelial cells and reduce TGFβ signaling in fibroblasts along with inactivation of neutrophil elastase [163-168]. The inhibitory actions of A1AT upon intracellular caspase-3 are attenuated by cigarette smoke [168].

TGFβ1 levels fell during RSO-dosing, with studies showing that it's signalling can be reduced by A1AT, while MMP-7 can cause its release from the extracellular matrix. TGFβ1 itself can enhance intracellular caspase-3 induced apoptosis with dying cells releasing further TGFβ1 [169]. Overall plasma TGFβ1 levels are poorly reflective of disease activity in IPF patients [141, 170-173].

Pink-1 and cytochrome C are both involved with activation of the intrinsic apoptotic pathway and mitophagy. Uncleaved Pink-1 accumulates in damaged mitochondria and marks them for lysosomal degradation via Parkin; while released mitochondrial cytochrome C activates the internal apoptotic pathway via caspase-9 [174]. Cytochrome C did show a significant negative correction with SCN that may reflect SCN effects upon the electron transport chain. Pink-1 may determine IPF susceptibility in older age via susceptibility to oxidative stress and apoptosis [107, 174, 175].

Overall the effects of RSO dosing upon the various mediators were very interesting, with summarised positive findings in figure 3 and a schematic diagram of their interactions in figure 4. Apart from SCN release by GL hydrolysis the other mediators were probably stimulated via TNF α; a cytokine normally activated by tissue damage, infection, inflammation or trauma, with the latter 3 causes not relevant in the medical staff. The significant increase in TNF α levels with RSO dosing suggests absorption of a GL metabolite capable of producing an intense inflammatory signal. This mediator is unknown, but skatole could be a contender in view of the apoptotic signal from caspase-3.

Interestingly, studies using a large range of L-trp metabolites injected into rabbit joints, showed only indole and skatole to produce joint inflammation; with skatole producing the most intense reaction with fibrosis that resembled a rheumatoid joint [176].

The increased levels of caspase-3 would support activation of the external apoptotic pathway through TNFα; with decreases in A1AT possibly reflecting its uptake by endothelial cells to inhibit caspase-3 activity. In addition, MMP-7 could stimulate VEGF activity to antagonise cellular apoptosis. The changes in caspase-3, VEGF, MMP-7 and A1AT would suggest involvement of the lung endothelial cells as all 4 mediators are particularly secreted by this tissue. Confirmation would require bronchial lavage studies with additional mediator assays in the light of these findings, including data from a larger group of subjects with a dose response to clarify the limited data from the medical staff.

Antibiotics have shown benefit in IPF, with azithromycin reducing hospital admissions by two thirds in a cohort of IPF patients previously reporting frequent infections [177]. Two other studies of cotrimoxazole showed reduced IPF exacerbation [178, 179]. Our 6 yr. longitudinal data from 53 patients (IPF + F-NSIP), showed an FVC decline of -60mls per year, with a SWT change of -27 metres/yr. Serum MMP-7 level were elevated upon entry at 11.54ng/ml but remained stable at 12months. We considered the actions of cotrimoxazole to represent blockade of the formyl peptide receptors which are considered tissue specific drivers of lung fibrosis; however effects on the microflora and GL metabolites may also need consideration in view of the veterinary data [179].

Interesting, IAA (Indole acetic acid), the precursor of skatole also shows adverse effects in animals with oxidative stress and DNA fragmentation described. Oral dosing in rats, produced anaemia with elevations in mean cell volume (MCV) and gamma-glutamyl transferase (GGT) from hepatocellular injury even at low serum levels (0.4µg/ml) [180]. Impairment of renal function with elevations in cardiac (CK-MB) and skeletal muscle enzymes (CK-MM) also occurred; along with histology showing reduced muscle fibre striations, necrotic nuclei and degenerative change. IAA withdrawal did not reverse all these changes and may have parallels to the myositis reported in the toxic oil syndrome and Eosinophilic Myalgic syndrome [181]. We have also reported unexplained elevations in MCV, GGT and monocytes in lung fibrosis patients recruited for our cotrimoxazole study; where liver ultrasound, vitamin B12,folate and viral serology were otherwise normal. Cotrimoxazole corrected these abnormalities relative to placebo patients. We concluded that oxidative stress and lipid peroxidation was responsible but the exact cause was obscure and IAA warrants consideration [182].

Glucosinolates are known to produce several other adverse effects in farm animals, many of which are also seen in IPF but are unexplained and warrant discussion here.

One of these, is the occurrence of hypothyroidism which is common in IPF without an explanation and affects men (13%) and women (28%) at rates well above the general population (<2% men, <9% women) [9, 183-186]. Hypothyroidism is well described in poultry and farm animals fed rapeseed products, where the GL metabolite of SCN blocks iodine uptake by the thyroid and the nitrile oxazolidin-2-thione product inhibits thyroid peroxidase synthesis of T_3 + T_4 hormones. Goiter can result that requires iodine supplements in livestock. The prevalence of hypothyroidism is also increasing in the UK population without explanation [187, 188].

Subclinical hepatic fibrosis is also described in a third of IPF cases with Fibroscan's showing asymptomatic increases in liver stiffness (mean 8.3±1.5kPa) relative to the normal range (4.8-5.5kPa). Liver function tests are normal and viral serology negative, however a 7 year follow up showed reduced survival in such cases: mean 33 months relative to 63 months for IPF patients with normal livers (HR 2.6) [189]. To date there is no explanation for this change with suggestions that IPF represents a "system-wide" fibrotic disorder [190, 191]. The veterinary thesis by Breeze reported hepatic and bile duct fibrosis at post mortems in the cattle with diffuse lung fibrosis [14]. Hepatic cholestasis also followed the toxic oil syndrome affecting 43% of patients acutely [43]. Brassica associated liver disease is well described in livestock with hepatic necrosis and fibrosis arising within months and correlating with GL intake [192-194]. Studies in pigs and rats suggest the nitriles and epithionitriles GL-products are the main cause [91, 194-196].

Up to 20% of IPF patients have type-2 diabetes, with released oxygen free radicals from the disease process considered to have adverse effects upon the pancreatic islet cells [9, 197]. However, 2 studies have linked a high dietary intake of nitriles to a modestly increased risk of incident type-2 diabetes. Both were prospective studies over 6-7 years that followed 2,139 and 104, 000 subjects respectively [198, 199]. A third larger US study, followed 401,000 patients for 22-28 years; and showed a modest positive association of diabetes with dietary glucosinolate intake. The causal GL component was not determined, but 2 servings per week increased risk by 3% [200].

Gastro-oesophageal reflux is described in >70% of IPF patients, with aggressive anti-reflux treatments and surgery ineffective against disease progression, leaving unresolved the assumed relationship [9, 197].

Nitriles can lower oesophageal tone causing sphincter relaxation and reduced gastric motility. This effect occurs through the release of nitric oxide (NO) from ingested nitrates or nitriles by gastric acid. NO is a diffusible gas that can permeate the gut mucosa affecting local motor transmission as shown in double blind studies, where it contributes to gastric motility disorders [201-204]. Nitriles are released from GL's by non-enzymatic autolysis at gastric acid pH to produce NO along with other nitrile products. This process is enhanced by SCN and polyphenols that are both abundant in Brassica plants and oils.

Mediastinal lymph node enlargement is described in a subset of IPF patients without known cause; biopsy studies show B cell follicles without germinal centres in a 25% of cases [205]. Lymph node enlargement was also reported by Breeze in a percentage of cattle that died from diffuse lung fibrosis; here the nodes showed haemorrhage with unexplained gas bullae but detailed histology was not undertaken [14]. The bovine "emphysema" may be relevant here but skatole is also a gas that may have escaped into the lung lymphatic channels.

Pulmonary hypertension is still poorly understood in IPF. Post mortems of fibrotic cattle by Breeze also described dilatation of the right ventricle and pulmonary artery trunk [14]. In IPF, surgical lung biopsies show parenchymal fibrosis to directly correlate with pulmonary vascular

change [11, 12, 100, 206, 207]. In the gut, L-trp is metabolised to serotonin (5HT) by the neuroendocrine cells, with first pass metabolism reducing blood levels by 30-80% following its absorption. Lung endothelial cells take up the remaining 5HT with a small fraction entering platelets [208].

Apoptotic lung endothelial cells will reduce their 5HT uptake, producing a marginal increase in blood 5HT levels that are still within the normal range. This has potent pulmonary vasoconstrictor effects and produces pulmonary hypertension in animal models [209]. Only one abstract reports plasma 5HT levels in lung fibrosis, showing levels at the top of the normal range especially for those with associated pulmonary hypertension [210]. Increases in 5HT affect local endothelial and vascular smooth muscle cell interactions, producing vascular smooth muscle proliferation in animals that may explain the direct histological links reported in IPF [211-213]. Pulmonary hypertension in IPF increases mortality considerably with limited treatment benefits, although positive findings are emerging for inhaled treprostinil [214]. A study in primary pulmonary hypertension confirms an accelerated turnover of plasma 5HT which was reduced by infusions of epoprostenol [215].

18% of IPF patients also have auto-antibodies with apoptosis a likely source of the raised circulating cell-free DNA [216]. 63% of IPF smokers or ex-smokers have autoantibodies relative to 30% of never smokers, with smoking a source of skatole which is already linked to a lifetime increased risk for rheumatoid disease [217-222]. Animal studies using case oil from the toxic oil syndrome did show mice to develop auto-antibodies (ssDNA + dsDNA) following oral dosing over 5weeks [224].

Telomere shortening occurs naturally with age and smoking and also constitutes a risk for carcinogenesis, diabetes and heart disease [8, 9, 115, 225-227]. In IPF, short telomeres appear an independent and unexplained risk factor for the disease. GL-metabolites (Especially indole-ITC, allyl-ITC and nitriles) can produce DNA damage with mutagenic effects in laboratory animals and cultured mammalian cells and this may need consideration [75, 228].

Nitrofurantoin (NF) is a highly lipid soluble antibiotic with low plasma concentrations and interesting parallels with IPF. Its long-term use could produce a fibrotic lung injury in some older patients on regular dosing over 6-70 months. This injury ceased upon drug withdrawal without lung tumours described [229, 230]. Animal studies showed NF to accumulate in type I and II alveolar cells, producing a dose dependant inhibition of the mitochondrial electron transport chain at complex 1+ 3. This was confirmed in lung and liver microsomes at therapeutic concentrations (1-2 µg/ml) and associated with oxidant release and glutathione depletion [231-232]. Generally the fibrosis arose in a subpleural and basal distribution akin to IPF, suggesting NF lung delivery via the gut lacteals and lymphatics.

There is now a much greater understanding of the lung lymphatics after 3-dimentional casting and electron microscopy has allowed previously unseen lymphatics connections to be identified [233]. This has shown the lung lymphatic flow to proceed in 2 different directions; with the peripheral and dorsal areas of the parietal pleura (Below the 4th intercostal space), draining caudally through the diaphragm to join the cisterna chyli. The cisterna chyli is the fusion of all gut lacteals and sits anterior to the L1 vertebra, before it ascends as the thoracic duct alongside the oesophagus to enter the left subclavian vein [234]. In contrast, all other lung lymphatics vessels drain up towards the hilum and enter the jugular veins.

Since the thoracic duct travels alongside the oesophagus, local reflux or cough induced diaphragm movements may affect chylous flow and produce pressure waves back to the cisterna with possible chylous reflux into the lung bases. Chylous entry to the subclavian vein will also follow dependant flow with increased delivery to the lung bases. Fats such as RSO have a long carbon chain (C22: 1) with absorption entirely restricted to the gut lacteals. If lipophilic products of GL metabolism are sequestered within the emulsified oil (Such a skatole), could the close proximity to the cisterna chyli allow chyle reflux into the lung base where the disease generally begins. Certainly this is likely for nitrofurantoin but needs consideration in IPF. Following a single lung transplant in IPF, fibrosis progresses in the native lung but does not arise within the transplant. This may result from characteristics of the donated lung, but normal lymphatic channels are completely disrupted following transplant and may influence in this outcome [235, 236]. Interestingly, poultry share the same adverse GL effects as other livestock, but do not develop lung injury with their avian lymphatic vessels known to be rudimentary [237]. The main limitations of this data are the small patient numbers limiting conclusion as to whether RSO-reductions could offer benefit in those predisposed to lung fibrosis. The widespread use of RSO makes recording food diaries difficult. For the medical staff a larger study with higher dosing including further mediators and also IAA and skatole would be of interest.

Table 1: The demographics of the UIP/IPF and fibrotic-NSIP patients.

Patient details	UIP/IPF (n=9)	F-NSIP (n=7)
Age (mean ± SEM) at start of diet	75.6 yr ± 2.16	71.0 yr ± 2.98
Male : Female ratio	5M : 5F	6M : 1F
Total duration of RSO-reduced diet (years)	4.8 ± 0.70	5.2 ± 0.36
No of patients with diabetes	1	0
Positive serum auto-antibodies	1 (Ds-DNA-13.0 IU/ml)	2 (rheumatoid factor 15 + 43 IU/ml) without clinical disease
Number of deaths	3	1
Cause of death	1 - Sudden death	1 - Post-viral pneumonia
	1 - Myelodysplasia	
	1 - Complication of a fall	
No of patients referred for anti-fibrotic drugs	2	3

Table 2: Shows first FVC (green background) before RSO reduced diet with annual FVC results absolute + % predicted for 9 UIP/IPF patients. Overall FVC change in ± ml/year is calculated by final FVC-FVC after dietary change commenced ÷ time in years with clinical status or treatment change shown.

Table 2 Year FVC (L) (FVC% predicted)	Patient 1 UIP/IPF	Patient 2 UIP/IPF	Patient 3 UIP/IPF	Patient 4 UIP/IPF	Patient 5 UIP/IPF	Patient 6 UIP/IPF	Patient 7 UIP/IPF	Patient 8 UIP/IPF	Patient 9 UIP/IPF
2014									2.09 (94%)
2015									2.24(100%)
2016								1.12 (59%) #	1.88
2017					3.2 (104%)	3.09 (91%)		1.21(63%)#	1.84
2018				2.54 (81%)	2.6(84%)	3.15(93%)	3.20 (103%)	1.10 #	1.86 (88%)
2019				2.3 # (76%)	2.8	2.98 (88%)	2.80 (91%)	1.15 # (55%)	1.87
2020	2.57 (115%)			- ¤	2.86 (93%)	2.8	2.42 (78%) #	0.80 #	1.71 (85%)
2021	2.4(107%)	2.4 (84%)	1.93 (105%)	1.9 # (63%)	2.71 (87%)	2.8	2.34 (75%) #	0.87 # (41%)	1.26Φ# (66%)
2022	2.35	2.1 (82%)	1.75 (97%)	1.9 (63%)	2.3 #ǂ (76%)	2.8*× (82%)	1.98* # @ (64%)	1.31# (66%)	0.88Φ#© (46%) died
2023	2.43 (109%)	2.01* (81%)	2.07 (125%)	Died ¤	1.68 ʘ# (56%)	2.5 + (78%)	2.85 (92%)	Died after fall	
FVC change ml/yr. ± % per yr.	+26ml/yr. (+0.6%)	-45 ml/yr. (-0.5%)	+ 160ml/yr . (+14%)	- 100ml/yr . (-2.5%)	-153ml/yr. (-13%)	-108ml/yr. (-2.5%)	+ 10ml/yr. (+0.2%)	+16ml/yr. (+0.5%)	-170ml/yr. (-6.7%)

Tertiary referral declined. ǂ spinal compression fractures, ʘ FVC limited as post-spinal procedure, Φ post 2nd and 3rd Covid-19 vaccine decline requiring oxygen. *Covid-19 infection, @ Pacemaker inserted. ¤ myelodysplasia diagnosed. × coronary stent inserted, © delayed agreement to referral. +Nintedanib commenced

Table 3: Shows first shuttle walk test (green background) for UIP/IPF patients + total HRCT scores in brackets. The mean change in shuttle walk (m) has been calculated (final SWT- SWT after dietary change ÷ number of years) to give the change as ± metres/yr. with total HRCT score shown.

Table 3 Year: SWT(M) (HRCT score)	Patient 1 UIP	Patient 2 UIP	Patient 3 UIP	Patient 4 UIP	Patient 5 UIP	Patient 6 UIP	Patient 7 UIP	Patient 8 UIP	Patient 9 UIP
2014									- (26)
2015				330 (26)		540 (12)			440
2016				300		-			570 (20)
2017				300	640 (15)	510		370 (17)	360
2018				310 (26)	580 (22)	560	250 (16)	260 (16)	370 (22)
2019				300	570(23)	500 (28)	190	340 (17)	350 (34)
2020	360 (9)			- (24) ¤	-	490	-	300	340
2021	360	520 (7)	140 (19)	-	560	- (29)	-	360	250Φ# (46)
2022	530 (17)	510	190 (19)	- (26)	500 ǂ (27)	520* × (30)	130 ∞* (15)	260 (16)	- Φ (69) Died
2023	430	- (7) *	140	Died ¤	320 ʘ	500+	160	Died after a fall	
SWT change m/yr.	+23m/yr.	-5m/yr.	0m/yr.	-6m/yr. but limited data from 2020	-45m/yr.	-4m/yr.	-15m/yr.	-18m/yr.	-27m/yr
HRCT score	+8	0	0	0	+12	+18	0	0	+43

- no SWT result. ¤ myelodysplasia with 20% blasts. Φ post-Covid-19 vaccine decline , #-tertiary referral declined ǂ Vertebral fractures. * Covid-19 infection. × coronary stent, inserted, ∞Pacemaker inserted , ʘ-back procedure, + nintedanib commenced.

Table 4: Shows first FVC (green background) before RSO reduction with annual FVC results absolute + % predicted for 7 F-NSIP patients. Overall FVC change in ± ml/year is calculated by final FVC-FVC after dietary change ÷ time in years, with clinical status or treatment shown.

Table 4* Year + FVC (L) +(% Predicted)	Patient A F-NSIP	Patient B F-NSIP	Patient C F-NSIP	Patient D F-NSIP	Patient E F-NSIP	Patient F F-NSIP	Patient G F-NSIP
2017	4.21 (96%)	4.2 (96%)			2.24 (101%)		
2018	3.62(83%)	3.45(78%)		3.72 (106%)	1.70(77%)	1.8 (47%)	2.3 (60%)
2019	3.70 (91%)	3.8 (91%)	2.0 (90%)	3.61(102%)	1.71	1.78(46%)	2.8 (73%)
2020	3.21	3.6	2.1	3.39 (96%)	1.73	1.76	2.7
2021	3.75	3.65 (83%)	1.7	3.33	1.8 (81%)	1.76	2.65 (70%)
2022	3.70* (90%)	3.72	1.8	3.33*+ (95%)	Died lobar pneumonia	1.49*+ (40%)	1.9 * (51%) ●
2023	3.71 (90%)	3.59 (81%)	1.8 (81%)	3.16 (90%)	-	1.46+ (39%)	0.8 + (21%)
FVC change in ml/per year (% change per Yr.)	+15ml/year ♦ **(+1.16%)**	+23mls/year (+0.5%)	-75mls/year (-2.2%)	-90ml/year (-2.4%)	+25ml/year (+1%)	-64mls/year (-1.4%)	-400ml/year (-10.5%)

*Covid-19 infection. + Nintedanib treatment referral, ● steroid trial 8 weeks requiring oxygen 4/l/min, + Nintedanib, ♦ lifetime smoking

Table 5: Shows first shuttle walk test (green background) for F-NSIP patients with HRCT scores in brackets. The mean change in shuttle walk (m) has been calculated by (final SWT- SWT after dietary change ÷ number of years) to give the change as ± metres/yr. with total HRCT scores shown.

TABLE 5 Year : SWT metres (HRCT score)	Patient A F-NSIP	Patient B F-NSIP	Patient C F-NSIP	Patient D F-NSIP	Patient E F-NSIP	Patient F F-NSIP	Patient G F-NSIP
2017	-	1020 (26)			-(24) ¥		
2018	330 (20)	1020 (23)		770 (22)	- (37)	- (10)	-
2019	-	1020 (20)	-	800 (22)	- (33)	300	290 (16)
2020	360 (25)	1020	- (16)	-	-	- (31)	420 (20)
2021	590 (35)	1020	-	-	-	300	- (25)
2022	570* (38)	1020 (27)	270 (20)	800 (38) *+	(54) died pneumonia	260 (29) *+	210 (48) *●+
2023	580	1020	250 (26)	630 virus		270	Too unwell +
SWT change metres/year	+36m/yr. ♦	0m/yr.	-10m/yr. (limited data)	-34m/yr.	No data	-6m/yr.	-20m/yr.
Change in HRCT score	+18	+1	+10	+16	+30	+19	+32

¥ no SWT due to osteoarthritis. - missed SWT. * Covid-19 infection. + nintedanib. ●steroid trial before nintedanib caused deterioration. + Nintedanib treatment. ♦ life time smoker

Table 6: Shows the results from the 10 measured mediators in the medical staff following a 14 day exclusion diet followed by 10 mls/day and 20 mls/day of rapeseed oil for 1 month each. It shows the means with confidence intervals and paired t-test results with significance <0.05.

Table 6 – Healthy Medical staff and ingested RSO effects on mediators

Mean serum levels n=8 (95%CI) NR=normal range	Cardiac Troponin ng/l NR <4Female <6Male	Thiocyanate Levels (SCN) µmol/l NR 33-44µmol/l	Caspase-3 pg/ml (NR <200)	Matrix metallo-protease-7 ng/ml (NR1.07-4.40)	Vascular endothelial growth factor pg/ml (NR 0-130)	TNF-α pg/ml (NR 5-27)	TGFbeta-1 pg/ml (NR 2-12,000)	α-1 Antitrypsin gm/L(A1AT) (NR 1.1-2.1)	Pink-1 Pg/ml (NR 320-700)	Cytochrome-C pg/ml (NR 27-212)
RSO free diet 2 weeks	1.04 (0.01,2.0)	50 (18,83)	155 (66,317)	1.72 (0.5,4.0)	38 (41,304)	15.4 (13,43)	6362 (5859,6865)	1.328 (0.98,1.66)	1743 (1460,2026)	58 (57,59)
RSO 10mls/d 2weeks	Not measured	59 (13,105)	225 (79,530)	3.43 (2.5,9.4)	193 (13, 374)	41.2 (9,92)	6083 (5519,6647)	1.326 (0.968,1.68)	1584 (1368,1800)	59.6 (57,63)
RSO 10mls/d 4weeks	1.90 (0.4,3.3)	73 # (31,115)	264 (161,690)	9.80 ■ (4.6,14.9)	201 (141,665)	28.8 (2,59)	5763 (4911,6615)	1.400 (10279,1.52)	1632 (1356,1915)	61.6 (53,63)
RSO free diet 2 weeks	1.13 (0.2, 1.9)	57 (34,81)	325 (150,501)	2.78 (1.8,3.7)	164 (31,359)	16.6 (0.74,31.5)	6356 (5673,7040)	1.280 (1.16,1.39)	2296 (703,3888)	70 (50,91)
RSO 20mls/d 4 weeks	2.35* (0.76,3.9)	76 ● (42,109)	466 + (270,662)	5.9 (0.39,10.3) 2 samples failed assay	406 X (89,704)	36.0 ϴ (6.7,65.2)	5748 (4307,7188)	1.150 ʘ (1.03,1.270)	1943 (1386,2500)	66 (48,84)

Paired T-Test significance at <0.05 with p values. *Cardiac Troponin (pre VS 4 Wk 20ml) p=0.038. # SCN (pre Vs 4Wk 10mls) p=0.0283
■ (MMP-7 (pre Vs 4Wk 10mls) p=0.0116 ●TC (pre Vs 4 Wk 20ml) p=0.0286. + caspase-3 (pre Vs 4 Wk 20ml) p=0.0046.
X VEGF (pre Vs 4 Wk 20mls) p= 0.045 . ϴ TNF-α (pre Vs 4Wk 20mls) p=0.036. ʘA1AT (pre Vs 4Wk 20mls) p=0.0024

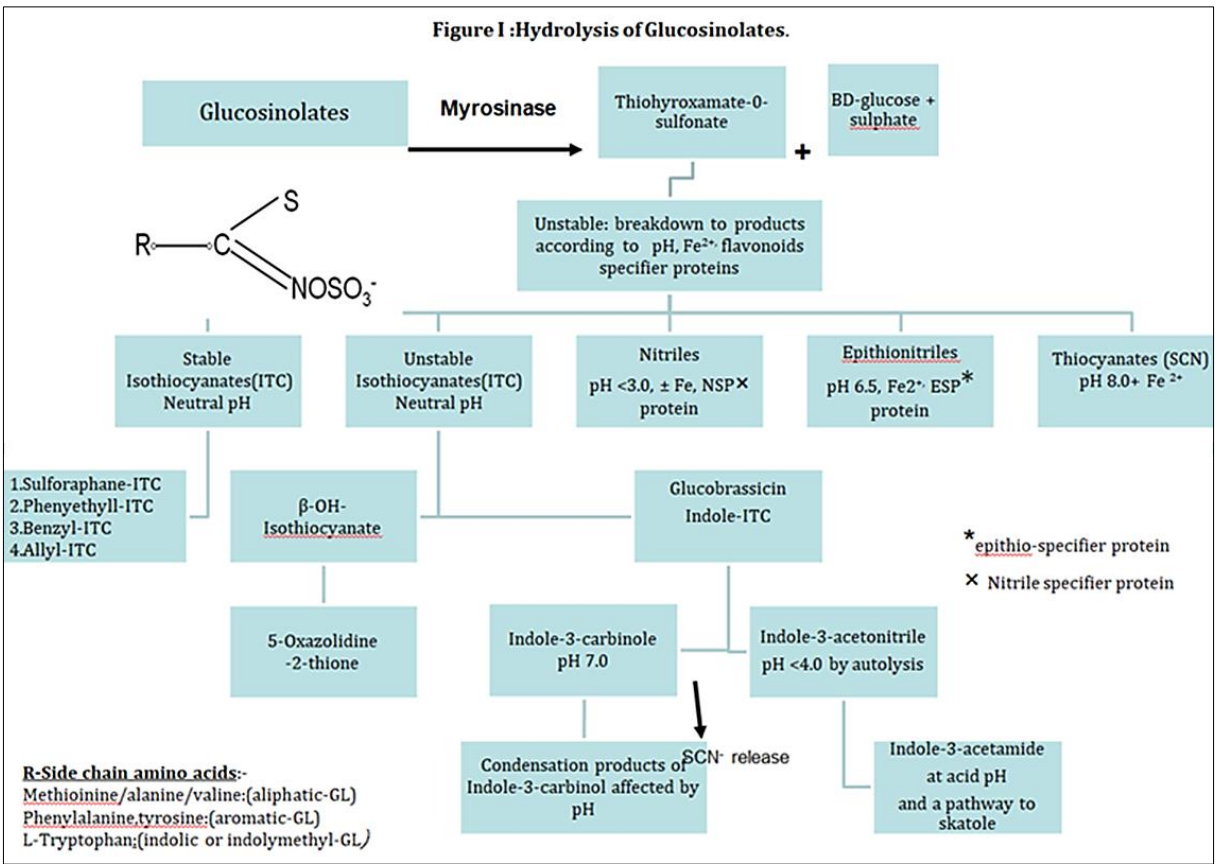

Fig 1: Enzymatic hydrolysis of Glucosinolates by myrosinase and their breakdown products.

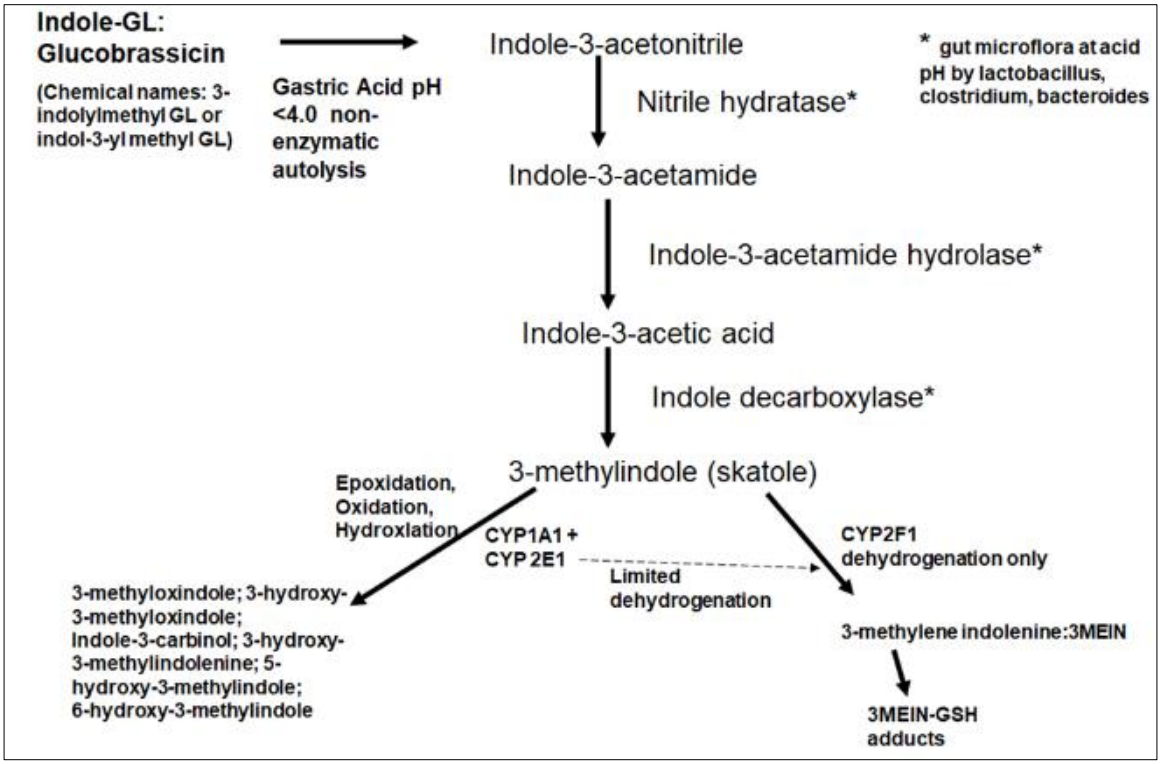

Fig 2: Shows the metabolite pathway of Glucobrassicin to skatole in mammals.

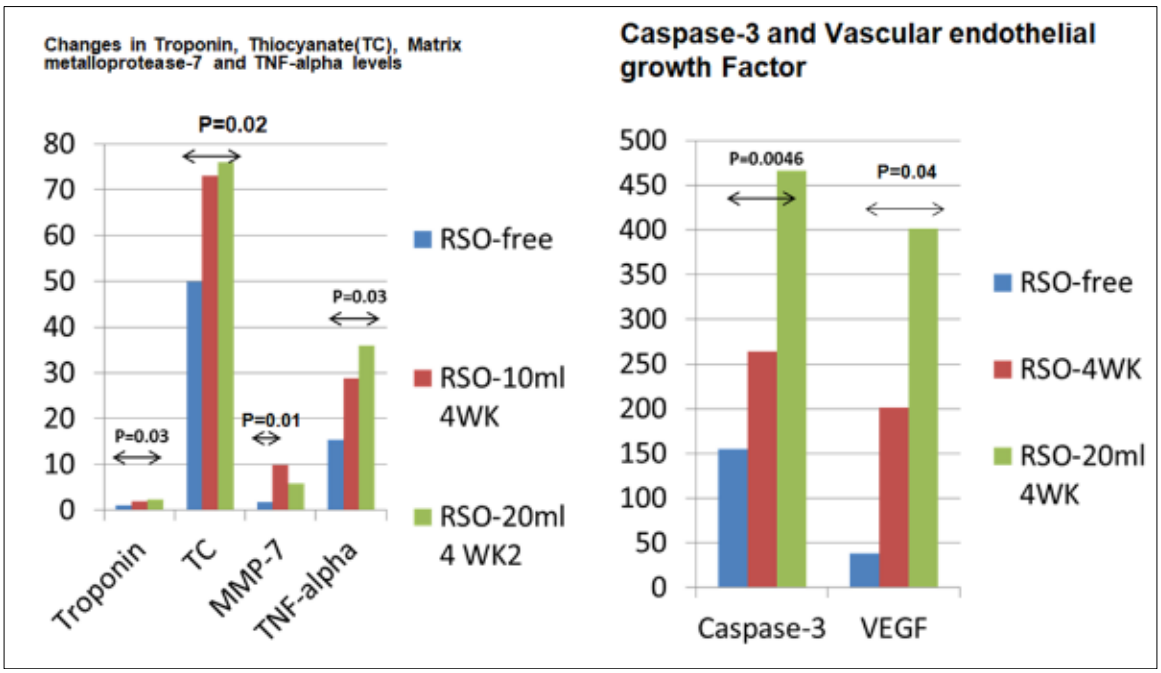

Fig 3: Graphically summarisation of mediator changes and p values during RSO-intake with the exception of A1AT.

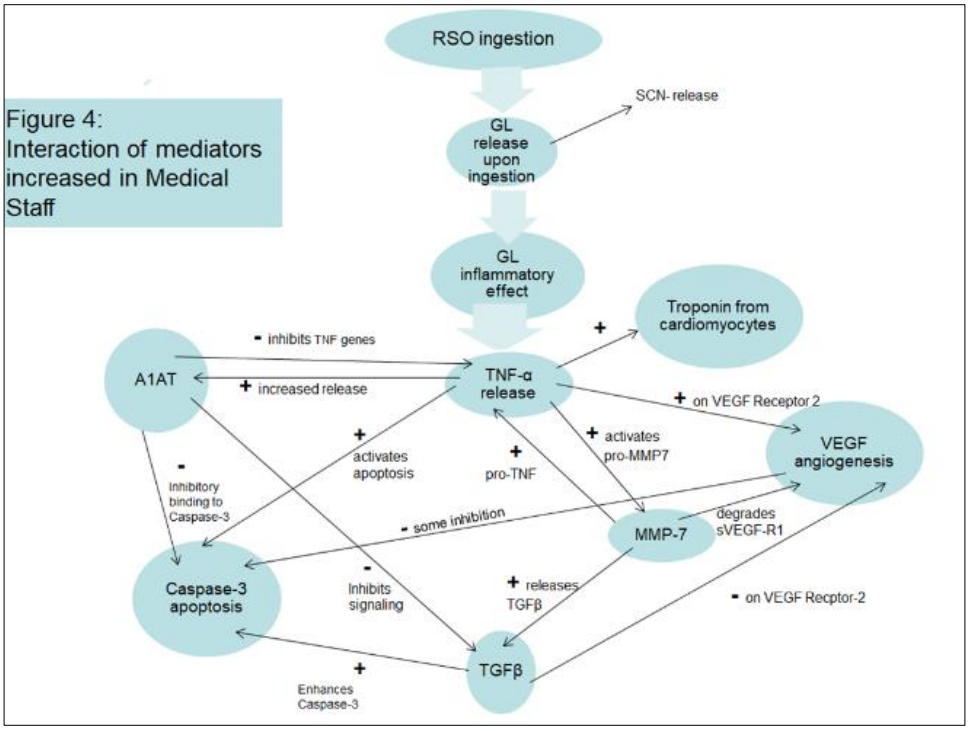

Fig 4: Schematic diagram of the discussed mediators and their known interactions with TNFα (tumour necrosis factor), VEGF (vascular endothelial growth factor), MMP-7(matrix metallopreases-7, AIAT (α-1 anti-trypsin), Caspase-3 and TGFβ (transforming growth factor beta).

Table 7: Mediator levels in the subject who took higher RSO doses (0-40mls/day) with results for thiocyanates, troponin I, caspase-3, TGFβ1 and PINK-1.

Condition	Thiocyanate (µmol/l)	Cardiac Troponin-I (ng/l)	Caspase-3 (Pg/ml)	TGFβ1 (Pg/ml)	PINK-1 (Pg/ml)
RSO-free diet	36	2.0	383	6615	1828
RSO-10ml/day (4 weeks)	38	2.6	660	5525	1826
RSO-20mls/day (4 weeks)	56	3.4	867	5625	2168
RSO-30mls/day (2 weeks)	49	3.5	952	6160	1830
RSO-40mls/day (2 weeks)	46	3.6	958	6460	1632

Conclusion

Idiopathic conditions will always have a cause or trigger that is ultimately recognised. For IPF, the search has continued since the 1980's, during which time the incidence and mortality has risen with a pressing need to understand the causal agent and affect a cure.

The veterinary data linking acute lung injury and diffuse lung fibrosis to forage brassica generated skatole is compelling, including the association with older and predominantly male animals. The spectrums of other GL effects in the animals are also seen in IPF, suggesting a link that may justify investigation. Overall the intake of Brassica vegetables in the human diet is unchanged with only seasonal variations; but GL exposure has changed following the increased use of Brassica containing oils. The timeline of this change is not incompatible with the appearance of IPF and the medical staff findings need explanation. Clearly the answer to these questions would require extensive epidemiological and clinical studies, including measurement of plasma 3MEIN and other mediators. Since PET scans show intense activity in IPF lungs, improvement after dietary change may give an early indication as to whether further intense research is justified.

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Declarations

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Details of kits and assays methods

Serum Cardiac Troponin-I (pg/ml) Measured in-house by our biochemistry lab using Architect Stat troponin-I 3P25 (Abbott Laboratories UK). A 2-step immunoassay with anti-troponin-I antibody-coated by paramagnetic micro-particles to which serum troponin-I binds occurs with Acridinium-labeled conjugates for a chemiluminescent reaction.CV % <3.2%, t½ of 2 hrs.

Measurement of plasma Thiocyanates (SCN). Plasma samples sent to the reference lab by courier with appropriate transport conditions. The samples had their protein precipitated by 10% tricholoracetic acid with the supernatant then analyzed for thiocyanate SCN^- spectro photometrically using an automated biochemical analyzer. The thiocyanate was chlorinated by hypochlorite and

quantified according to König reaction using iso-nicotinic acid and 1, 3-dimethyl-barbituric acid. This measured free SCN^- group. Potassium thiocyanate was used as the standard. Detection limit of 5 µmol/L, in-house CV < 2.4%. Analysis performed at HSE's Health & Safety Laboratory Buxton, Derbyshire, SK17 9JN, UK. (J Prakt Chem 1994; 69:105-37).

Caspase-3 An ELISA kit (Cat No #K4221 Bio Vision Incorporated) for human Caspase-3 via sandwich ELISA assay using biotin-detection antibody and streptavidin-conjugate system with the microplate read at 450 nm. No dilution of samples. Sensitivity of <0.188 ng/ml with CV <6%.

Matrix Metalloproteases-7 assay by quantitative sandwich immunoassay ELISA (R&D systems catalogue No. DMP700), using a micro-plate coated with human MMP-7 with optical density reading. Serum samples were diluted 1 in 2 with lowest detection range was 0.005ng/ml, CV <4%.

Human VEGF Immunoassay pg/ml A solid phase Immunoassay ELISA (R&D systems catalogue DVE00) for human VEGF-A165a and VEGF-A165b. Detection range 5-1000pg/ml and CV <5%. t½ 30minutes No dilution of the samples.

TNFα Biotinylated anti-human TNFα antibody coated microplate with a HRP-conjugated streptavidin with colorimetric analysis at 450nm. (ELH-TNFa RayBio ELISA). Sample dilution 1 in 2. Intra-assay CV <10%.

TGFβ1 Human ELISA (Cat #E4508 BioVision Incorporated) with HRP-conjugated streptavidin with colorimetric analysis at 450nm. The sample dilutions were 1 in 10. Detection range 31-2000pg/ml. Intra assay CV<10%.

Serum Cytochrome C:ELISA kit (#E4286-100 BioVision incorporated). A microplate coated with biotin detection antibody for cytochrome C and a HRP-streptavidin conjugate for color intensity reading at 450nm. No dilutions of the samples. Sensitivity <46pg/ml and CV <8%.

Serum Pink-1 (PTEN-induced putative Kinase-1)

ELISA kit Cat No: EH15183. Wuhan Fine Biotech Co Ltd. Enzyme-linked Immuno- assay using pre-coated microplate with biotin conjugated anti-Pink-1 antibody and HRP-Streptavidin for colorimetric absorbance at 450nm. Sample dilution was 1 in 2. Sensitivity was < 0.094ng/ml; CV <8%.

Serum levels of alpha-1 Antitrypsin (A1AT)

Siemens healthineers (CE0197) an immune complex reaction using N-anti-serum to Alpha-1 Antitrypsin to bind serum sample (diluted 1 in 20) producing a immunochemical complex with scattered light proportional to the concentration in the sample. Detection range <0.047 g/l and CV %< 3%.

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