

## **A Review on Metformin with Emphasis on its Inhibiting Effects on Cancer**

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## 1. Introduction

The anti-diabetic biguanide metformin (N,N-dimethylbiguanide hydrochloride) has pleiotropic properties<sup>1-3</sup>. Importantly, cancer suppression is increasingly being recognized as one of these properties<sup>4-9</sup>. These properties tend to be mainly AMPK (5'-adenosine monophosphate activated protein kinase) related but can be unrelated<sup>7;10-14</sup>. Most studies report that the majority of effects are achieved through inhibition of complex 1 of the electron transfer chain<sup>13;15;16</sup> hence increasing the AMP:ATP ratio. Energy depletion is detected by AMPK<sup>13;17;18</sup> which inhibits gluconeogenesis, lipogenesis, cellular proliferation and stimulates

uptake of glucose by muscle by inhibition of the mammalian target of rapamycin (mTOR)<sup>19;20</sup>. mTOR is a protein kinase which can bind to either of two substrates, one includes Raptor, making it rapamycin sensitive (mTORC1 - mTOR complex 1), the other includes Rictor, making it rapamycin insensitive (mTORC2 - mTOR complex 2)<sup>12</sup> (Fig 1).

Metformin use has the dual outcomes of firstly, reducing insulin resistance, increasing glucose sensitivity hence reducing diabetic morbidity and mortality<sup>21;22</sup> while secondly and potentially more importantly suppressing tumourigenesis<sup>7;9</sup>. Other biguanides have been used such as phenformin (N-phenylethylbiguanide) but exhibit side effects such as a high risk of lactic acidosis which obviate use<sup>23;24</sup>.

Type 2 diabetes incidence is increasing worldwide<sup>25</sup> because of lifestyle and an aging population as age increases risk<sup>26</sup>. Hence, diabetes-related cancers are also increasing in frequency. In Europe 55.4 million people have diabetes with a yearly cancer incidence of 3.2 million and in the US similar data are 23.6 million and 1.4 million<sup>27</sup>. However, the etiology of these cancers is uncertain because obesity usually accompanies type 2 diabetes along with other factors which cause cancer<sup>27;28</sup>. Nevertheless, metformin's pleiotropic mechanisms mean that the etiology of these cancers is relatively unimportant.

Possible AMPK-unrelated effects of metformin include the inhibition of RAG-GTPases (Ras-related GTPases) which thus inhibit mTOR<sup>12</sup>. Furthermore, metformin activates the tumour suppressor LKB1 (serine-threonine kinase - liver kinase B1) directly or indirectly. Directly, metformin may activate LKB1 which then activates downstream AMPK<sup>29</sup> or indirectly through metformin's activation of AMPK, when LKB1 is needed for AMPK activity<sup>30</sup> except in certain cells when AMPK is activated by cytosolic Ca<sup>2+</sup> increase, hence calmodulin-dependent kinase kinase-β (CaMKKβ) which phosphorylates the AMPK a subunit like LKB1 and can act without<sup>31</sup> or synergistically with AMP<sup>32</sup>. Other tumour suppressors activated by AMPK through metformin include tuberous sclerosis complex 1 (harmartin) and tuberous sclerosis complex 2 (tuberin) (TSC 1 & 2)<sup>33</sup>.

Inflammation has been purported to lead to carcinogenesis<sup>34;35</sup>. Inflammation is inhibited by metformin's actions on the mTOR pathway, consequently leading to the inhibition of nuclear factor-κB (NF-κB) and Interleukin 6 (IL-6) through feedback loops<sup>36;37</sup>.

Observational studies have shown metformin use to reduce the risk of several different cancer types but studies are varied in method and standard and often other medications are involved<sup>38;39</sup>. Adjuvant trials are ongoing but *in vivo* and *in vitro* studies have shown that metformin may have effects at all stages of cancer development from initiation<sup>40;41</sup> to metastasis<sup>42</sup>. Hence, metformin's effects on healthy subjects need to be addressed.

This review aims to examine the evidence for metformin's cancer suppressing efficacy, the mechanisms by which it acts, the levels at which it works and whether metformin should be administered as a cancer preventive medication<sup>43</sup>.

## 2. General outcomes of metformin use

### a. Different tissues

Metformin acts through AMPK by increasing energy creating mechanisms and decreasing energy consuming effects, which result in reducing blood glucose and insulin levels. This has various effects in different tissues<sup>22;44</sup>. Metformin's actions through AMPK suppress hepatic gluconeogenesis and lipogenesis but induce glucose uptake, glycolysis and fatty acid oxidation. In adipose tissue metformin similarly suppresses lipogenesis but also prevents insulin resistance, lipotoxicity, glucotoxicity and

inflammation and stimulates glucose uptake, fatty acid oxidation and insulin sensitization. The drug has similar effects in muscle cells but its effects on insulin resistance and insulin sensitization are more important as muscle cells account for more than 80% of insulin-stimulated glucose uptake<sup>44</sup>.

### **b. Type 2 diabetes mellitus and obesity**

Type 2 diabetes mellitus is characterized by hyperglycaemia due to glucose intolerance and hyperinsulaemia because of insulin resistance. This differs from Type 1 diabetes mellitus where hyperglycaemia is caused by failure of the pancreatic Islets of Langerhans  $\beta$  cells to secrete insulin, primarily because of autoimmune breakdown and where exogenous insulin must be taken. Type 2 diabetes does not usually require exogenous insulin but either control through diet or by taking anti-glycaemic medications such as metformin. Type 1 diabetes mellitus is relatively low in frequency compared with Type 2 which constitutes over 90% of cases worldwide<sup>25</sup>.

Insulin resistance in Type 2 diabetes is caused by a lack of tissue responsiveness to insulin possibly because of several purported causes: hyperlipidaemia particularly concerning diacylglycerol which triggers novel protein kinases C causing impaired insulin signaling<sup>45</sup> and/or insufficient insulin binding in muscle cells<sup>46</sup>; inflammation caused by NF- $\kappa$ B activation and cytokine production<sup>47,48</sup>; regulation of DGAT1 (diacylglycerol (DAG) acyltransferase) which controls triglyceride synthesis<sup>49,50</sup>; baseline leptin levels<sup>51,52</sup>; mitochondrial dysfunction<sup>53</sup> and/or faulty GRK2 (G protein-coupled receptor kinase 2) modulation of insulin responses<sup>54,55</sup>. Anfosso *et al.* (1993)<sup>56</sup> showed that insulin receptor numbers were reduced in human hepatoma cell line Hep G2 when exposed to high concentrations of insulin. Outcomes are insufficient glucose elimination and increased hepatic gluconeogenesis resulting in increased glucose levels<sup>22</sup>. Compensatory insulin secretion (hyperinsulaemia) results in  $\beta$  cell insufficiency because of destruction or exhaustion of these cells, hence hyperglycaemia. Resulting microvascular complications include retinopathy, nephropathy and neuropathy and increased risk of cardiovascular disease, peripheral arterial disease and cerebrovascular disease<sup>22</sup>.

### **c. Carcinogenesis caused by hyperinsulaemia, bioavailable IGF-I and IGF-II and sex hormones**

Chronically high insulin levels and diabetes are associated with colorectal, endometrial, pancreatic, liver, postmenopausal breast and bladder cancers and non-Hodgkin Lymphoma (NHL)<sup>27,57</sup>. Raised insulin levels also cause inhibition of hepatic synthesis and circulating levels of sex-hormone binding globulin (SHBG)<sup>58</sup>. This increases the levels of bioavailable oestradiol in males and females<sup>59</sup> and testosterone, which increase the risk of sex-hormone related cancers such as that of the breast and endometrium<sup>60</sup>. In females it also increases bioavailable testosterone but in males leads to reductions in total-testicular testosterone production. In the severely obese, reduced SHBG levels cause static or even a reduction in bioavailable testosterone<sup>59</sup>. Increased insulin levels can increase ovarian and adrenal androgen synthesis leading to premenopausal polycystic ovary syndrome (PCOS) in the genetically susceptible<sup>59</sup>.

Adiposity accompanies type 2 diabetes which affects the synthesis and bioavailability of endogenous sex hormones (oestrogens, androgens and progesterone) by adipose-tissue secretion of sex-steroid metabolising enzymes (aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase [17 $\beta$ -HSD]) that promote sex hormone synthesis from precursors secreted by the gonads or adrenal glands<sup>61,62</sup>. Moreover, BMI has been shown to be directly related to oestrone and oestradiol circulating levels<sup>59,63</sup>. Aromatase is upregulated by cortisol, insulin and increased oestradiol and leptin concentrations which further upregulate aromatase through cyclic amplification<sup>64</sup>.

Evidence indicates that these adipose-related sex-hormone bioavailability alterations promote the development of several cancers including postmenopausal breast cancer and endometrial cancer<sup>65,66</sup>.

and also possibly prostate cancer<sup>66</sup>. Female gallbladder cancer has also been partially attributed to adipose-related oestrogen increases<sup>67</sup>. A combination of adipose-related sex-hormones may act synergistically in carcinogenesis<sup>66</sup> however, although prostate cancer induction is purported to be related to androgen metabolism, the association with excess adiposity is disputed<sup>61</sup>.

Increased insulin levels lead to increased levels of free Insulin-like Growth Factor 1 (IGF- I) via different mechanisms, for instance: insulin displaces IGF- I from Insulin-like Growth Factor 1 binding protein (IGFBP- I) thus decreasing binding protein levels and increasing IGF-1 bioavailability<sup>68</sup>; stimulation of IGF- I and IGF- II release through hepatic hormone upregulation<sup>69;70</sup> and suppression of IGFBP levels<sup>71</sup>. IGF- I binds to the receptor IGF- IR with high affinity and to the insulin receptor (IR) with low affinity and *vice versa* for insulin in breast cancer cells<sup>72;73</sup>. Hybrid receptors (or heterodimers) also bind both IGF- I and insulin<sup>73</sup> and the ratio between homo- and heterodimers may contribute to insulin resistance<sup>73;74</sup>.

The increase in free IGF- I leads to various cancers *in vitro*, *in vivo* and in humans through various pathways via its receptor (IGF-1R) (Fig 1) (see below), such as colorectal<sup>57;75;76</sup> breast<sup>72</sup>; prostate<sup>75</sup> and endometrial cancer<sup>77</sup>. IGF-II binds to IGF-IR and also the IR-A isoform of the insulin receptor with high affinity to promote mitosis in foetal cells and, importantly, in cancer cells<sup>78</sup>. The IR-B isoform is mainly expressed in differentiated insulin target cells<sup>78</sup>. The *IGF2* gene is imprinted and loss of imprinting leads to proliferation and diseases such as Wilm's tumour, colon cancer<sup>79</sup> hepatocellular carcinoma<sup>80;81</sup> and breast cancer<sup>81</sup> due to increased levels of free IGF-II which then bind to IGF-IR<sup>82</sup>.

#### d. Metformin's cancer suppressing effects

Metformin lowers insulin levels by its activation of AMPK which inhibits translocation of mTORC2 (also known as CRTC2 [CREB regulated transcription coactivator 2]) to the nucleus therefore inhibiting transcription of gluconeogenesis genes by CREB (cAMP-response element binding protein)<sup>20;21</sup> (Fig 1) (see below). This anti-gluconeogenic action happens in both type 2 diabetic patients and hyperinsulaemic non-diabetic patients leading to anti-proliferative effects<sup>83</sup>. Lowering of insulin levels by metformin is achieved through increasing insulin sensitivity and also decreasing insulin secretion. Metformin decreases insulin secretion from the  $\beta$ -cells of the Pancreatic islets via AMPK action<sup>77;84</sup> moreover, autophagy in  $\beta$ -cells, which contributes to T2D, has also been shown to be ameliorated by metformin<sup>85</sup>. Insulin sensitivity is improved in human hepatoma cell line (Hep G2) by metformin possibly by several post-receptor mechanisms including restoring receptor substrate tyrosine kinase activity, enhancing glucose transport<sup>56</sup> and induction of insulin-regulatable glucose transporter (GLUT-4) translocation in rat soleus muscle<sup>86</sup> and human skeletal muscle and adipose cells involving AMPK, serine/threonine protein kinase (AKT) and Protein kinase C, zeta (PKCzeta)<sup>87</sup>. AMPK-mediated downregulation of the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) which negatively regulates insulin sensitivity was thought to be the mechanism involved in metformin's insulin lowering and sensitizing in preadipocyte 3T3-L1 cells<sup>88</sup>.

Uncoupling protein 2 (UCP2) downregulates oxidative phosphorylation in response to oxidative stress<sup>89</sup>. There is evidence that UCP2 also regulates insulin secretion as UCP2 deficient mice become hypoglycaemic because of increased insulin secretion<sup>90</sup> whereas insulin levels are low when UCP2 is overexpressed<sup>91</sup> therefore dysregulation of UCP2 levels may lead to  $\beta$  cell dysfunction and type 2 diabetes development<sup>92</sup>. Metformin's suppression of complex 1 of the electron transfer chain causes increases in UCP2 levels in adipocytes<sup>89</sup> thus this may be a mechanism for metformin's actions on insulin secretion.

A high fat diet is an important factor leading to hyperglycaemia and type 2 diabetes<sup>47;93</sup>. Fatty acid oxidation is reduced and lipid accumulates in skeletal muscle of obese individuals<sup>94</sup> also in aging mice<sup>26</sup>.

This was attributed to reduced muscle mitochondrial content possibly because of loss of transcription factor regulation of mitochondrial biogenesis in obese individuals<sup>94,95</sup>. However, metformin was found to reduce fatty acid transport by the FA transporter (FAT/CD36), ceramide and diacylglycerol (DAG) levels and therefore hyperglycaemia was decreased in Zucker rat skeletal muscle<sup>96</sup>. Metformin also increased PGC-1 $\alpha$  (Peroxisome proliferator-activated receptor gamma coactivator 1  $\alpha$ ) levels and mitochondrial biogenesis possibly through AMPK activation in rat skeletal muscle<sup>97</sup>. Metabolic defects in the AMPK pathway due to lipid oversupply was found in rat liver<sup>98</sup>. Metformin may be expected to ameliorate this problem.

IGF-I and II levels are lowered through metformin's ability to lower insulin levels and its effects on binding proteins<sup>22</sup>. This is further facilitated by metformin's activation of AMPK which leads to phosphorylation of the inhibitory site Ser789 of IRS-1<sup>99</sup> (Fig 1). Metformin's action on insulin levels also lowers sex hormone levels because of restoration of normal SHBG production in the ovary and liver. Sex-hormone levels are further lowered by metformin's effect on weight and waist-to-hip ratio<sup>100</sup> because lowered adiposity also inhibits aromatase production. Metformin may also reduce aromatase levels in breast tissue therefore reducing oestrogen levels locally through its activation of AMPK and inhibition of TORC2 which is known to increase aromatase expression<sup>101;102</sup>. This may decrease the risk of post-menopausal breast cancer.

### 3. Molecular pathways and inhibitory effects of metformin

The pleiotropic effects of metformin indicate that many metabolic pathways are affected by its actions. The cell's metabolism is inherently complex where there is constant change and movement, fluctuation, associations and dissociations, passage in and out of the nucleus, positive and negative feedback loops and mechanisms etc. thus any written text or diagrams are insufficient to convey this complexity. The relative importance and certainty surrounding different pathways and processes related by different studies are difficult to determine and some pathways are better researched than others. Moreover, it is difficult to compare *in vivo* and *in vitro* studies because of different responses<sup>103</sup>. Nevertheless, the text and diagrams will attempt to convey metformin's actions and effects on major pathways within all this complexity and uncertainty.

## 4. AMPK dependent pathways

### I. AMPK activation

AMPK regulates key specific metabolic processes in different tissues, for example, liver, muscle and adipose tissue<sup>104</sup> (see above). AMPK pathways contain several tumour suppressor genes and proto-oncogenes<sup>33</sup> making them important therapeutic targets (Fig 1). Mutations in proto-oncogenes are activating mutations and those in tumour suppressor genes are inactivating mutations.

Most studies have found that metformin activates AMPK by increasing the AMP:ATP ratio through inhibiting oxidative phosphorylation<sup>13;89;105;106</sup>. The drug mildly inhibits the electron transfer chain at Complex I<sup>89;105;106</sup> while normalizing H<sub>2</sub>O<sub>2</sub> (a product of superoxide, a reactive oxygen species [ROS]) emission by blocking reverse electron flux<sup>107</sup> and without affecting forward electron flow and skeletal muscle respiration in obese Zucker rats<sup>107</sup>. However, some studies postulate that AMPK is activated by metformin without altering the AMP:ATP ratio<sup>29;108;109</sup>. This was tested in isolated rat hearts and it was found that AMPK acted as a cellular energy sensor and always reacted to increased cytosolic AMP levels (due to metformin and phenformin application) but not total AMP or AMP:ATP ratio<sup>110</sup>. AMPK reacts by activating energy producing processes and reducing proliferative processes<sup>3;13;21;110</sup> (Fig 1).

AMPK is activated upstream by LKB1 (also known as STK11 [Serine/threonine-protein kinase 11]) and also mediates the activity of LKB1<sup>30;111</sup>. Metformin may activate LKB1 itself through steps involving the generation of the superoxide anion and reactive nitrogen species which activate c-Src (a proto-oncogene), phosphatidylinositol 3-kinase (PI3K), Phosphoinositide-dependent kinase-1 (PDK1) and Protein kinase C, zeta (PKCzeta) which then phosphorylates LKB1 which activates downstream AMPK<sup>29;112;113</sup> (Fig 1). Metformin may also activate LKB1 indirectly through its activation of AMPK, when LKB1 is required for AMPK activity. LKB1 is located in the nucleus and when phosphorylated translocates into the cytosol where it phosphorylates AMPK<sup>29</sup>.

Metformin's direct or indirect activation of LKB1 may additionally activate its tumor suppressive effects. LKB1 is a master kinase, which, besides phosphorylating AMPK, also phosphorylates 12 related kinases involved in cell growth, metabolism and polarity<sup>104;112</sup>. Mutation of the *LKB1* gene leads to Peutz-Jeghers syndrome which is an autosomal inherited dominant condition in which patients are LKB1 deficient and are at risk of developing several cancer types<sup>111</sup> and may be the result of the failure to activate AMPK<sup>114</sup>. However, mutations of the *LKB1* gene have been found in lung adenocarcinoma and squamous cell carcinomas<sup>115</sup> in people without Peutz-Jeghers syndrome. Deficiency of *LKB1* was found to be more important in the initiation, differentiation and metastasis of lung adenocarcinoma than mutations of other suppressor genes in mouse models<sup>115</sup>. Other cancers that may be caused by mutations of *LKB1* are head and neck cancer<sup>116</sup>, pancreatic and other cancers probably at a low level<sup>117</sup>. *LKB1* mutation promotes and progresses 20% of cervical cancers in combination with the Human Papilloma Virus (HPV)<sup>118</sup>. The cancer suppressing effects of wild-type LKB1 on these cancers are stimulated by metformin's actions. The anti-tumour effect of LKB1 was found to be further enhanced in HeLa cells by WDR6 (WD repeat protein 6) which promotes cell cycle arrest synergistically in association with LKB1<sup>119</sup> (Fig 1). LKB1 and AMPK have conserved roles in cell polarity which when disrupted is implicated in carcinogenesis. This may not apply to all tissues as *LKB1* deletion did not affect polarity or organization in every tissue in mice.<sup>117</sup>

Ouyang *et al.* (2010) have suggested a novel mechanism by which metformin may activate AMPK using isolated skeletal L6 cells. Rather than the AMP:ATP ratio being increased because of inhibition of oxidative phosphorylation by metformin, AMP levels are increased by metformin's attenuation of AMP deaminase (AMPD) which normally breaks down AMP<sup>120</sup> (Fig 1).

## II. Inhibition of the mTORC1 pathway

Insulin, IGF-I and IGF-II bind to IR/IGFR which then bind to IRS-1 (insulin receptor substrate 1) which subsequently transmits signals to Ras (small GTPase) and PI3K (phosphatidylinositol 3-kinase) along the pathway to mTORC1 (Fig 1). Mutations in the Ras proto-oncogene subfamily (K-Ras, H-ras and N-Ras) are implicated in most human tumours<sup>33</sup> and the *PI3CA* oncogene encodes the catalytic subunit 110a of PI3K and is frequently mutated in solid human tumours<sup>121</sup>. Besides PI3K other downstream effectors of Ras share an area of homology called the Ras binding domain (RBD)<sup>33</sup> see below (Fig 2). The PI3K pathway is a well documented oncogenic pathway and mutations in the pathway are implicated in up to 30% of human cancers<sup>122</sup>.

A feedback loop exists (shown in Figure 1 in red) whereby mTORC1 and ribosomal protein S6 activation leads to inhibition of IRS1 at Ser<sup>639</sup> thus inhibiting downstream effectors including AKT<sup>99;123</sup>. Activation of AMPK by metformin inhibits this feedback loop, however, this is compensated for by the inhibition of IRS-1 by the phosphorylation of the inhibitory site at Ser<sup>789</sup> which limits downstream signalling<sup>99</sup>. Activation of TSC1 & 2 leads to the inhibition of RHEB (Ras homologue enriched in brain – GTPase) and mTORC1 signalling<sup>99</sup> (Fig 1)

The tumour suppressors NF1 (neurofibromin 1) and PTEN (Fig 1) are often mutated in human cancers<sup>124;125</sup>. Metformin's activation of AMPK prevents tumourigenesis in a mouse model due to loss of PTEN<sup>124</sup> and therefore possibly in humans. This may also be the case for NF1 loss as an *in vitro* study has shown using rapamycin, a mTORC1 inhibitor<sup>125</sup>.

Caveolins are proteins necessary for the formation of Caveolae which are plasma membrane invaginations (50-100nm) that regulate signal transduction<sup>126</sup> and lipid transport<sup>127</sup>. Caveolin-1 (*Cav-1*) can act as a tumour suppressor or promoter depending upon the cell type<sup>126;128</sup> (Fig 1). For instance, it acts as a tumour promoter in urothelial bladder cells and prostate cells<sup>126</sup> and as a tumour suppressor in the ovary<sup>126</sup> and in breast tissue<sup>129</sup> where 16-20% of breast tumours display mutations<sup>128</sup>. *Cav-1* acts as an mTORC1 pathway controller although it is uncertain which part of the pathway it activates, possibly PI3K. Loss of *Cav-1* promoted activation of PI3K/AKT pathway in oestrogen positive breast tumours<sup>130;131</sup> but it activated the AKT/mTOR pathway to promote disease progression and metastasis in human renal cell carcinoma (RCC)<sup>132</sup>.

Importantly, *Cav-1* has been shown to act as a promoter of androgen insensitive prostate cancer in *in vitro* and *in vivo* studies<sup>133;134</sup>. This happens by inhibition of PP1 and PP2A phosphatases which leads to increased PDK1, AKT and Erk1/2 activity and cell survival<sup>133</sup> and increased *Cav-1* expression is correlated with ability to form tumours in a mouse model<sup>134</sup>.

The contrasting activities of *Cav-1* may be because different peptide domains have different functions. Phosphorylation at Tyr14 and Ser80 and P132L mutations cause a change in topography by converting it from a membrane protein to a secretory product which removes its suppressor activities and also gives it autocrine/paracrine tumour promoting properties<sup>126</sup>. There is evidence that there are two opposing roles for *Cav-1* in different cancer phases: the suppressing effects of *Cav-1* may be lost and upregulation may be an acquired feature during metastasis<sup>126</sup> where stromal carcinoma-associated fibroblasts (CAFs), stretch due to restructuring mediated by *Cav-1* which allows invasion<sup>135</sup>. Conversely, *Cav-1* expression was shown to reduce EMT characteristics and promote pancreatic cancer cell differentiation by restoring E-cadherin<sup>136</sup>.

NF-1 binds to *Cav-1* scaffolding domain to regulate cell growth and differentiation through Ras, FAK (focal adhesion kinase) and AKT pathways<sup>137</sup>. PDK1 (3-Phosphoinositide dependent protein kinase 1) the next downstream effector, is a master kinase and also a nuclear translocator<sup>138</sup>. It phosphorylates AKT (serine/threonine protein kinase) also known as Protein Kinase B (PKB), which phosphorylates GSK-3 (glycogen synthase kinase-3) and many other substrates that control cell growth, metabolism, cell cycle and survival<sup>33</sup> some of which are shown in Fig 3.

AKT activates MDM2 (murine double minute protein) which is a negative regulator of the tumour suppressor p53<sup>139</sup> (Fig 1). AKT itself is also activated by mTORC2 and requires this phosphorylation to be fully activated<sup>12</sup> (Fig 1). However, both mTORC1 and mTORC2<sup>20</sup> are inhibited by metformin induced AMPK actions (Fig 1).

GSK-3 controls cell cycle through phosphorylation of cell cycle regulators such as Cyclin D1 (Fig 1). It also phosphorylates transcription factors such as SREBP1 (Sterol regulatory element binding transcription factor 1)<sup>33</sup> (Fig 1). Usually phosphorylation by GSK-3 results in impaired function so that deactivation by AKT means enhanced transcription<sup>33</sup>. Therefore, metformin's actions on AMPK and thus AKT inhibition result in GSK-3 activation and inhibition of transcription leading to cell cycle arrest and fatty acid oxidation (Fig 1).

GSK-3 also phosphorylates TSC 2 in a manner dependent on AMPK priming phosphorylation<sup>140</sup> (Fig 1). The TSC2 subunit of the TSC 2 & 1 complex is activated by AMPK via metformin<sup>141</sup>. This improves TSC complex stability thus TSC inhibition of mTORC1 through TSC 2 inhibition of RHEB. This reduces GLUT1 glucose transporter levels and glucose uptake<sup>142</sup>.

AMPK activation was also found to inhibit mTORC1 directly by phosphorylating Raptor (Fig 1) at two serine sites in MEFs (mouse embryonic fibroblasts)<sup>143</sup>. The relative input of TSC2 and Raptor in inhibiting mTORC1 will be anticipated to vary in different tissues and will depend on the expression of these proteins in different tissues and crosstalk between the various pathways involved<sup>144</sup>.

AMPK is a metabolic control point where inhibition of TSC2 by AKT is balanced by activation of TSC2 and inhibition of Raptor (thus mTORC1) by AMPK involving various pathways and crosstalk with energy requirement and use being central to the process. Metformin intervention can be used to balance this process towards energy requirement.

### III. Inhibition of other Ras pathways

There are several activating and inactivating mutations in these pathways (Fig 2). Ras (itself an oncogene) activates the Raf pathway which via MEK activates ERK. ERK phosphorylates many cell growth and cell cycle mediators and also RSK also known as P70 S6K (ribosome protein S6 kinase) which contribute to cell growth and proliferation. The Ras/MEK/ERK pathway promotes transcription of VEGF (vascular epidermal growth factor) via phosphorylation of transcription factors Sp-1, HIF-1a (hypoxia-inducible factor – 1a) and possibly activator protein-1 (AP-1)<sup>145</sup>. When PI3K was inhibited by Wortmannin, endothelin-1 (ET-1)-induced ERK2 activation was attenuated in human ovarian carcinoma cells<sup>146</sup>. Metformin, through inhibition of the mTOR pathway, may be projected to act similarly (Fig 2 and Fig 4).

Other RBDs are shown on Figure 2. Although the only demonstrated effectors that are activated in human cancers are Raf and PI3K, lack of RalGDS (Ral guanine nucleotide dissociation stimulator), PLC-e (phospholipase C family member) or Tiam1 (T-cell lymphoma invasion and metastasis 1) in mice reduced tumour incidence in Ras-dependent tumorigenesis models<sup>33</sup>. RASSf1 (Ras association domain-containing protein 1) interacts directly and indirectly with Ras although its functions in mediating its effects are unclear.<sup>147</sup> Some studies have postulated that it may be a tumour suppressor<sup>148</sup>.

Rho GTPases are involved in many processes so that signal aberrations are implicated in various aspects of cancer<sup>149</sup>. During cancer initiation, Rho GTPase may inhibit apoptosis so imposing cell longevity, abnormal growth and loss of polarity. Alteration of adhesion proteins may cause invasion and ability to metastasize<sup>149</sup>. The actions of metformin may reduce the activation of these critical effectors (Fig 2).

### IV. Inhibition of other AKT pathways

AKT inhibits apoptosis in several ways by phosphorylating the apoptotic BAD (k Bcl-2-associated death promoter)<sup>150;151</sup> and inactivating caspases and FOXOs (Forkhead family of transcription factors) which effect apoptosis<sup>152</sup>. Figure 3 shows how metformin may inhibit AKT effectors.

Metformin has been shown to reduce vascular inflammatory effects by inhibiting NF- $\kappa$ B via the inhibition of the PI3K–AKT pathway<sup>36</sup>. Metformin inhibited NF- $\kappa$ B nuclear translocation and I $\kappa$ B degradation (Fig 3) in vascular smooth muscle cells<sup>36</sup>. *In vivo*, metformin has been shown to inhibit I $\kappa$ B phosphorylation and NF- $\kappa$ B activation in the aortic vessel wall and decrease high-sensitivity C-reactive protein (hs-CRP) level in rabbit serum<sup>153</sup>.

Lipid accumulation in the livers of mice fed on a high-fat diet leads to sub-acute hepatic inflammation through NF- $\kappa$ B activation and cytokine production leading to hepatic and systemic insulin resistance<sup>47</sup>. Mechanisms may entail a positive feedback loop involving NF- $\kappa$ B and IL-6 (Fig 3). Mediation of NF- $\kappa$ B via Src oncoprotein activation in breast cells results in a positive feedback loop involving the transcription of Lin-28 (microRNA inhibitor processing factor) which inhibits Let-7 microRNA epigenetic modifier (which usually prevents IL-6 effects)<sup>37</sup>. Thus IL-6 levels rise which increase STAT3 (transcription 3 transcription factor) which activates microRNAs miR-21a and miR181b-1<sup>154</sup>. These inhibit PTEN and CYLD tumour suppressors thus increasing NF- $\kappa$ B activity<sup>154</sup>. The outcome is malignant transformation, self-renewal of cancer initiating cells (cancer stem cells) and increases in cell motility and cancer cell growth<sup>37</sup>. Activation of IL-6 also increases NF- $\kappa$ B levels which forms the positive feedback loop<sup>37</sup>. IL-6 increases inflammation<sup>47</sup> (see below). A concurrent slower feedback mechanism involves the activation of Ras through deactivation of Let-7 which again activates NF- $\kappa$ B<sup>37</sup>. Metformin may eliminate these feedback loops by reversing all the processes involved (Fig 3) and recent supporting evidence suggests that reduced Let-7 levels are responsible for cancer stemness and that metformin via AMPK may inhibit Lin-28 and boost Let-7 levels<sup>155</sup>. NF- $\kappa$ B transcribes cytokine-induced inflammatory genes such as GRO $\alpha$ , COX2 and VEGF<sup>37</sup> and adhesion molecule genes<sup>156</sup> which are again inhibited by metformin via AMPK<sup>156</sup> (Fig 3).

Activation of NF- $\kappa$ B and STAT3 was found to depend on upstream signaling via PI3K and AKT but not mTOR in the development of Myc<sup>E $\mu$</sup>  B-cell lymphomas in mice<sup>157</sup>. Anomalous activity has been revealed in these pathways for various types of B-cell neoplasms<sup>157</sup>. Moreover, the PI3K/AKT pathway, activated by TNF or IL-1, was not found to activate NF- $\kappa$ B in human endothelial cells despite inhibiting apoptosis and only had a minor role in the pro-inflammatory responses due to this activation<sup>158</sup>.

Increased AKT activity leads to increased nitric oxide (NO) production via endothelial nitric oxide synthase (eNOS) but is inhibited by metformin (Fig 3). High insulin production impairs the PI3K-AKT-NO signaling pathway leading to the detrimental vascular effects of hyperinsulinaemia.<sup>159</sup> This pathway controls many complex effects of NO on vascular smooth muscle cells (VSMC) including VSMC relaxation, capillary recruitment and inhibition of adhesion molecule expression<sup>159</sup>.

#### V. Inhibition of mTORC1 effectors

mTORC1 phosphorylates its substrate P70 S6K via the master kinase PDK1 (Fig 4). P70 S6K regulates the multiple phosphorylation of 40S ribosomal protein S6 *in vivo*<sup>160</sup>. This has been shown to be inhibited by rapamycin<sup>161;162</sup> (an mTORC inhibitor) which prevents the cell progressing through the G1 phase of the cell cycle<sup>161</sup> (Fig 4). As metformin was shown to inhibit P70 S6K in Tamoxifen resistant, oestrogen deprived, MCF-7 breast cancer cells<sup>163</sup> and epithelial ovarian cancer cells<sup>9</sup> metformin would be expected to act similarly to rapamycin. However, metformin suppressed HER2 oncoprotein overexpression by attenuating P70 S6K activity directly in a AMPK-independent manner in breast cancer cells<sup>164</sup>.

Metformin's actions possibly reduce mTORC1 phosphorylation of 4EBP1 (Eukaryotic initiation factor 4E binding protein-1) which heterodimerizes and thus inactivates the cap-binding protein eIF4E (Eukaryotic initiation factor 4E)<sup>165;166</sup>. This negates its binding to the cap-structure of mRNA and therefore protein translation<sup>165</sup> (Fig 4).

Insulin and IGF-1 promote angiogenesis e.g. in diabetic retinopathy and neovascularisation during hypoxia by stimulating HIF $\alpha$  and VEGF through the PI3K/mTOR pathways<sup>167</sup>. Metformin was shown to inhibit HIF $\alpha$  expression induced by insulin and IGF-1 through the mTOR pathway in retinal epithelial ARPE-19 cells via AMPK<sup>167</sup> (Fig1 and Fig 4) and also through decreased ATP production and oxygen consumption in FDF rat kidney tubules and human renal proximal tubular epithelial cells (HRPTECs)<sup>168</sup>.

In melanoma cells it was found that ET-1, by activating its GPCR (G protein coupled receptor), provokes tumorigenesis and progression through activation of the PI3K/TORC1 pathway<sup>169</sup> (Fig 1). This causes the inhibition of PHD2 (HIF-prolyl hydroxylase domain-2) thus promotion of HIF-1a stability, neovascularisation and tumour cell division<sup>169</sup>. Similarly, DNA replication induced by ET-1 and transactivation of the EGF receptor in colorectal cancer cells was shown to be inhibited by pertussis-toxin sensitive G protein, PI3K and PKC antagonists<sup>170</sup>. Metformin, via AMPK, has been found to disrupt crosstalk between IR/IGFR and GPCR in pancreatic cancer cells<sup>19</sup>, and would be predicted to inhibit ET-1-induced colorectal cancer (Fig 4).

#### VI. Inhibition of the mTORC2 pathways

There appears to be fewer studies devoted to mTORC2 than mTORC1 pathways. However, the complex is an important part of metformin's anti-cancer effects and is part of the network of pathways which link the complexes (Fig 1). For instance, mTORC2 was thought to be rapamycin insensitive<sup>171</sup> nevertheless, although rapamycin cannot bind to mTORC2 directly, prolonged use disrupts mTORC2 assembly in 20% of cancer cell lines and therefore strongly inhibits AKT signaling<sup>165</sup> thus impeding mTORC1 signaling in a feedback loop which exists for metformin's actions via AMPK (Fig 1) which may also have similar effects on mTORC2. Moreover, a trial involving Everolimus, an mTOR inhibitor, indicated inhibition of both complexes in early breast cancer patients<sup>123</sup>.

Besides metformin's indirect effect on cancer risk through AMPK's phosphorylation of mTORC2 thus preventing the transcription of gluconeogenesis genes<sup>20</sup> (see above) (Fig 1), there are more direct effects which affect cell morphology and motility. Control of the cytoskeleton possibly involves mTORC2 modulation of actin polymerization, as inhibition of any component of the complex causes similar alteration to cell morphology and adhesion<sup>172</sup>. Modulation involves PKCa (a master kinase) which has various functions depending on cell type and is also involved in cytoskeleton organization<sup>173</sup>. The small GTPases (Rho, Rac and CDC42) (Fig 1) are also involved, which suppress actin insufficiency caused by downregulation of mTORC2<sup>171</sup>. Downstream of PKCa, Rac1 modulates lamellipodia formation and migration and RhoA has been shown to activate transcription factor (AP-1) in T cells<sup>173</sup>.

#### VII. Inhibition of cell cycle pathways

Metformin's postulated cell cycle inhibitory effects take place via several cell cycle pathways including those downstream from AMPK; LKB1 synergistic activation with WDR6<sup>119</sup>; mTORC1; and AKT effects on MDM2 and GSK-3<sup>174</sup> pathways (Fig 1).

Metformin activates AMPK which induces phosphorylation of p53 on serine15 which initiates cell-cycle arrest<sup>175</sup> and there is evidence that AMPKp is located at the chromosomes during mitosis indicating modulation<sup>176</sup>. Proliferation was inhibited by metformin in cultured breast cancer cells<sup>177</sup> and prostate cancer cells<sup>178</sup> through G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. This happened in breast cancer cells through Cyclin D1 downregulation and CDK inhibitory binding effects on Cyclin E/CDK2 (Fig 1) preventing progression from G<sub>1</sub> into S phase<sup>177</sup>. However, in some cancer cells (e.g. MDA-MB-231) there is loss or downregulation of CDK inhibitors rendering them metformin insensitive<sup>177</sup>. In ER positive and negative and *erbB2* normal and abnormal cancer cell lines, cell cycle arrest was demonstrated to be partial and metformin dose-dependent<sup>179</sup>. Similarly, in triple negative (TN) breast cancer cell lines where metformin blocked G<sub>1</sub> phase of the cell cycle<sup>6</sup>.

Cell cycle progression was again blocked by metformin in the G<sub>0</sub>/G<sub>1</sub> phase, possibly through AMPK activation, without significant cell death in low-density C6 rat glioma cell line cultures whereas in dense cultures caspase-dependent apoptosis occurred<sup>180</sup>.

AKT is pivotal to some proposed cell cycle pathways as it positively controls G<sub>1</sub>/S cell cycle progression through inactivation of GSK-3 $\beta$ , thus increasing Cyclin D1, inhibiting FOXO (Forkhead family transcription factor) and TSC2 leading to a reduction of CDK p27<sup>kip1</sup><sup>174</sup>. It was hypothesized that the PI3K pathway may also be important in the G<sub>2</sub>/M transition and that activation may lead to defects in DNA damage checkpoint control<sup>174</sup>. One study<sup>181</sup> of medulloblastoma (the most common solid paediatric cancer) in mice found that TSC stabilizes CDK inhibitor p27, preventing cell cycle progression from G<sub>1</sub> into S phase.

PI3K/AKT and ERK pathways (Figs 1,2 &3) were inhibited by the activity of sulphoraphane (a compound found in cruciferous vegetables)<sup>182</sup> which may be comparable to metformin action. This was found to activate FOXO transcription factor leading to cell cycle arrest and apoptosis in pancreatic cancer cells<sup>182</sup>.

AKT is controlled in different directions by mTORC1 and mTORC2 (Fig1). It was found that rapamycin-mediated mTORC2 inhibition caused greater AKT-dependent cell cycle G<sub>1</sub> phase arrest than mTORC1 attenuation, resulting in strong downregulation of Cyclin D1 and upregulation of p27<sup>183</sup> which may be the situation for metformin. S6K1 and 4E-BP1/e1F4E pathways were hypothesized to be key mediators of mTOR dependent cell-cycle control<sup>184</sup> (Fig 4).

#### **VIII. Inhibition of fatty acid synthesis**

AMPK activation inhibits ACC (acetyl-CoA carboxylase) which increases fatty acid oxidation<sup>185</sup>. Metformin was shown to activate AMPK hepatocytes taken from treated rats which inhibited ACC activity<sup>3</sup> and more recently in human adipose tissue<sup>186</sup>. This induces fatty acid oxidation and reduced expression of lipogenic enzymes, for example fatty acid synthase (FAS) and spot-14 (S14)<sup>3</sup> (Fig 1). Expression of SREBP1 and other lipogenic mRNAs and proteins were attenuated<sup>3</sup> (Fig 1). Downregulation of SREB1 also happens due to the AKT inhibition and thus increased GSK-3 activity (see above) (Fig 1).

Activation of AMPK by metformin reverses glucose-induced repression of PPAR $\alpha$  (peroxisome proliferator activated receptor- alpha) (Fig 1) and specific PPAR $\alpha$  genes in pancreatic  $\beta$  cells and isolated rat islets where control of fatty acid metabolism is important for  $\beta$ -cell viability and function<sup>187</sup>. As high concentrations of fatty acids are toxic to  $\beta$  cells<sup>187</sup> metformin may be  $\beta$ -cell protective. AMPK activated PPAR $\alpha$  has also been shown to control fatty acid oxidation and skeletal muscle<sup>188</sup>.

#### **IX. Inhibition of protein synthesis independently of mTOR**

Inhibition of protein synthesis through AMPK activation by metformin is not only through mTOR but also through inhibition of elongation factor-2 (eEF2) which mediates the main translocation step in protein synthesis<sup>189-191</sup> (Fig 1).

## **5. AMPK-independent pathways**

### **I. RAG GTPases**

Metformin and phenformin were found to suppress mTORC1 signaling independently of AMPK and TSC by inhibition of RAG GTPases in MEFs, Drosophila Kc167 cells and HEK293T cells (human embryonic kidney cells)<sup>12</sup> (Fig 1). RAG GTPases are able to induce mTORC1 translocation to a perinuclear compartment containing RHEB. It was found that in growing cells mTOR located into large perinuclear aggregates but when amino acids were removed it diffused throughout the cytoplasm which was reversed when amino acids were replaced<sup>12</sup>. A later study<sup>192</sup> demonstrated that RAG GTPases associate with a complex named Ragulator which mediates mTORC1 aggregation and association with RHEB at the lysosome surface.

## II. AMPK inhibition studies

Metformin was found to activate the AMPK pathway, block the G<sub>0</sub>/G<sub>1</sub> cell cycle phase while not inducing apoptosis in prostate cancer cells (DU145, PC-3 and LNCaP cells). However, inhibition of the two catalytic sub-units of AMPK using siRNA (small interfering RNA) did not prevent the anti-proliferative effects of metformin through a strong decrease in cyclin D1 levels<sup>178</sup>. Nevertheless, as the siRNA did not completely abolish AMPK protein expression<sup>178</sup> this may indicate that there may have been some residual effect. The same study group found that metformin up-regulated REDD1 (regulated in development and DNA damage responses 1) gene expression, which inhibited mTOR activity and induced cell cycle arrest in a p53-mediated fashion independently of AMPK in LNCaP prostate cancer cells<sup>193</sup>. Similarly, metformin (not AICAR) inhibited UPR in renal proximal tubular epithelial cells in an AMPK-independent fashion<sup>194</sup>.

A similar study was done on MCF-7 breast cancer cells where AMPKα1 subunit was inhibited. In this study it was found that metformin action was blocked indicating that AMPK was essential to its growth suppressive effects<sup>195</sup>. These opposing results may indicate differences in the metabolism of the two different cancers as indicated by observation study results (see above) and further evidence that *Cav-1* is a tumour promoter in prostate cancer<sup>126</sup> and suppressor in breast cancer<sup>129</sup>.

When AMPK was inhibited using Compound C, metformin still caused cell-cycle arrest in the S and G<sub>2</sub>/M phases and induced apoptosis by activating caspases 3/7, down-regulating Bcl-2 and Bcl-xL expression and up-regulating Bax and Bad in epithelial ovarian cancer cell lines<sup>196</sup>.

## III. In vivo studies

In mouse liver tissue metformin activated AMPK and inhibited the mTOR pathway whereas in lung tissue AMPK was not activated but inhibition of IGF-IR/IR, AKT, ERK and mTOR took place<sup>7</sup> again illustrating different tissue responses to metformin. The authors of the study considered this to be lack of direct response by lung tissue to metformin because of insufficient uptake and/or because of decreasing circulating levels of growth factors because of metformin's effects on other tissues<sup>7</sup>.

## IV. AMPK- and LKB1-independent hypoglycemic effects

Metformin was found to inhibit hepatic gluconeogenesis in mice lacking AMPK in the liver<sup>197</sup>. The drug was also found to decrease expression of genes encoding for the catalytic subunit of glucose-6-phosphatase (G6Pase) while cytosolic phosphoenolpyruvate carboxykinase (pepck) gene expression was unaffected in wild-type AMPK-deficient and LKB1-deficient hepatocytes leading to amplified inhibition of glucose production. This correlated with reduced intracellular ATP concentration in a dose-dependent manner. Thus, metformin was found to inhibit hepatic gluconeogenesis in an LKB1- and AMPK-independent manner via decreased hepatic energy status<sup>197</sup>. Similar results were revealed in rat hepatoma cells<sup>198</sup>. Recently, Metformin was shown to inhibit glucagon by increased AMP levels which inhibit adenylate cyclase, leading to decreased cyclic AMP (cAMP), PKA, CREB and gluconeogenesis in mouse hepatocytes<sup>199</sup> (fig 1).

## V. AMPK-independent HIF-1a suppression

Although metformin activated AMPK, it decreased ATP production and oxygen consumption rates independently of AMPK, which AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) and rapamycin failed to do, to suppress HIF-1a expression in ZDF rat kidney tubules and human renal proximal tubule epithelial cells (HRPTECs)<sup>168</sup>.

## VI. Solid tumours

Secretory and trans-membrane proteins are folded and modified in the endoplasmic reticulum (ER)<sup>200</sup>. Under stress conditions, UPR (unfolded protein response) allows cells to survive through regulation by X-box binding protein-1 (XBP-1) transcription factor<sup>200</sup>. PI3K subunits have been shown to interact with

spliced XBP-1 stimulated by insulin via IR<sup>200</sup>. Biguanides, including metformin, were able to modify UPR transcription during glucose deficiency in solid tumours (colon cancer, fibrosarcoma, stomach cancer, renal cell carcinoma) and induce significant cell death<sup>201;202</sup>. Moreover, the LKB1-AMPK pathway was demonstrated to be unnecessary for this to happen<sup>201</sup>.

## VII. Other Effects

Metformin is known to cause vitamin B12 deficiency and B12 deficiency is also known to inhibit cancer growth. Hence, some reports give this as one mechanism for metformin's inhibitory effects on cancer<sup>203</sup> and also enhanced response to chemotherapy<sup>204</sup>.

Metformin may act AMPK-dependently to reduce cardiac hypertrophy<sup>205</sup> and AMPK-independently to induce protein phosphatase 2A (PP2A) which reduces tau phosphorylation in primary cortical neurons of transgenic embryos<sup>206</sup>. Hyperphosphorylated tau has a role in the formation of neurofibrillary tangles in Alzheimer's Disease<sup>206</sup>.

## 6. Antioxidant actions

Studies have shown that oxidative stress contributes to cancer progression and metastasis and these can be reduced by powerful antioxidants<sup>207</sup>. Metformin has antioxidant properties as it reduces ROS<sup>107</sup>. It has been proposed that ROS (particularly hydrogen peroxide) from adjacent breast cancer cells downregulate Cav-1<sup>129</sup> in CAFs which in turn increases eNOS due to decreased co-precipitation with Cav-1<sup>129;208</sup>. Consequently, NO levels increase leading to mitochondrial dysfunction because of DNA damage, uncoupling and increased ROS levels.<sup>129</sup> Through the "bystander effect" a positive feedback loop is established when these ROS effect DNA damage leading to genomic instability and random mutagenesis and increased ROS production in the cancer cells affecting CAFs. The oxidative stress affecting the CAF mitochondria leads to mitophagy, hence aerobic glycolysis, secretion of nutrients and lactate and by the "Reverse Warburg Effect" leads to mitochondrial biogenesis in the adjacent cancer cells and oxidative metabolism<sup>129</sup>. Secreted lactate and ketones may then increase acetyl CoA levels which may lead to increased histone acetylation thus gene transcription. Thus epigenetics and energy metabolism could drive breast cancer outcome rather than gene profile *per se* and metformin's effects on energy metabolism would ameliorate this<sup>209</sup>. Metformin was also shown to restore Cav-1 activity in these breast stromal fibroblast co-cultures by its actions<sup>129</sup> conversely, Cav-1 was shown to be necessary for metformin's inhibitory effects on the IGF-1 pathway in NSCLC cells<sup>210</sup>. It would be expected that metformin's pleiotropic effects through inhibition of the mTOR pathway and other pathways detailed in this review would also contribute its effects.

Oxidative stress may be reduced by metformin's activation of AMPK resulting in FOXO3 and the antioxidant thioredoxin (Trx) upregulation thus ROS inhibition<sup>211</sup>. Metformin attenuated oxidative stress-induced apoptosis by reducing and or normalizing electron chain ROS production and by preventing loss of cytochrome c and NADH due to inhibition of mitochondrial permeability transition pore (PTP) opening in kidney endothelial cells<sup>2</sup> and in human microvascular endothelial cells (HMEC)<sup>212</sup>.

Metformin brought about a reduction in high fat-induced cardiomyocyte death probably through ceramide synthesis and caspase-3 inhibition<sup>213</sup>. However, it was found that at high concentrations metformin caused proton and lactate accumulation (lactic acidosis) leading to cell damage independent of caspase-3<sup>213</sup>.

## 7. Inhibition of inflammation

Inflammation is an immune response to physical, psychological and or oxidative stress which triggers the NF- $\kappa$ B pathway<sup>214</sup>. In acute inflammatory episodes NF- $\kappa$ B has an inhibitory effect on cancer eliminating transformed cells but when activated constitutively it is pro-tumorigenic<sup>214</sup>. Karin (2008)<sup>35</sup>, using mice, proposed a mechanism for the connection between inflammation and cancer. He proposed that two types of cells are involved: inflammatory cells such as macrophages, dendritic cells and/or neutrophils of the lamina propria and, in the case of colitis-associated cancer (CAC), intestinal epithelial pre-malignant cells. In inflammatory cells, pathogens and/or pro-inflammatory cytokines (e.g. TNF $\alpha$  or IL-1) enter the inflammatory cell, which, through MyD88 adapter protein, activate IKK $\beta$  which mediates transcription of NF- $\kappa$ B. Production of inflammatory cytokines such as IL-6 is then upregulated (see above and Fig 3). The IL-6 produced then activates STAT-3 in intestinal epithelial pre-malignant cells causing proliferation. NF- $\kappa$ B is also activated which upregulates survival genes such as Bcl-X<sub>L</sub><sup>35</sup>.

Inflammatory processes influence tumorigenesis and metastasis even in cancers that do not have their etiology in inflammation such as breast and prostate cancers. For instance, prostate metastasis involves solid tumour infiltration of lymphocytes and upregulation of receptor activator of NF- $\kappa$ B (RANK) and its ligand (RANKL)<sup>215</sup>. Metformin was shown to decrease RANKL expression in ovariectomized rats<sup>216</sup>.

STAT3 is a complex oncogene<sup>217</sup>. Cells that contain activated STAT3 secrete immunosuppressive factors and it contributes to lymphoma cell immune response evasion and also tumour progression<sup>217</sup>. This oncogene has been proposed as being the link between inflammation and cancer<sup>34</sup>. Here, more complex feedback mechanisms are postulated as taking place within a microenvironment including intrinsic STAT3-dependent autocrine feedback loops in neoplastic epithelia and extrinsic interactions between tumour (myeloid cell), inflammatory (lymphocyte) and stromal cells<sup>34</sup>.

Metformin may be predicted to inhibit these processes through the inhibition of AKT which is one of the upstream activators of IKK (Fig 3) and, as discussed above, in relation to the IL-6/NF- $\kappa$ B/STAT3 positive feedback loop<sup>37</sup>. In support of this, STAT3 knockdown in TN breast cancer cells enhanced metformin's enhancement of growth inhibition and apoptosis<sup>218</sup> and metformin was shown to be more effective than the insulin secretagogue Repaglinide, in reducing biomarkers of inflammation and endothelial dysfunction in a cross-over study of T2D non-obese subjects<sup>219</sup>.

Another study<sup>220</sup> named HIF-1 $\alpha$  as the link between inflammation and carcinogenesis. Where IL-6 upregulates NF- $\kappa$ B through the mTOR pathway (or independently) leading to increased transcription of COX-2 protein which upregulates HIF-1 $\alpha$  thus VEGF and carcinogenesis<sup>220</sup>.

Metformin (through AMPK) has been shown to inhibit cytokine-induced NF- $\kappa$ B in human umbilical vein endothelial cells (HUVECs)<sup>156;221</sup>, lipopolysaccharide (a component of bacterial cell walls)-induced proinflammatory genes in mouse macrophages<sup>222</sup> and increase survival in endotoxaemic mice<sup>223</sup>. Anti-inflammatory action was also demonstrated as metformin attenuated aortic atherosclerotic progression in rabbits (see above)<sup>153</sup>. Metformin affected release of both pro- and anti-inflammatory cytokines and reduced toxic molecule production in an AMPK-independent manner in rat microglia<sup>224</sup>.

IL-6 levels were found to be modulated by IL-6R gene polymorphisms in metabolic syndrome<sup>225</sup> which may affect the processes discussed above.

## 8. Immune cell activation

Metformin was shown to increase fatty acid oxidation and CD8<sub>T</sub><sub>M</sub> – cell generation in mice and improved the efficacy of an experimental anti-cancer vaccine although the mechanism was not known<sup>226</sup>. Moreover, the immunogenerative effect of biguanides was also demonstrated when phenformin (and Clofibrate) was given to breast cancer patients after radical mastectomy. It resulted in delayed sensitivity to DNCB (dinitrochlorobenzene), tuberculin, and candidin, increased T-lymphocyte count and improved lymphocyte blast transformation reaction<sup>227</sup>.

## 9. Effects on longevity and telomerase

Biguanides (metformin, buformin and phenformin) significantly increased the life-span of rats<sup>228</sup> through mechanisms detailed above i.e. prevention in age-related deterioration in: glucose and insulin metabolism; fertility (due to the hypothalamo-pituitary complex sensitivity to negative feedback inhibition); and tumourigenesis and apparently to a greater degree than calorie restriction<sup>229;230</sup> (Fig 1) and genetic manipulation<sup>228</sup>.

Long-term treatment with metformin was shown to increase the life-span of spontaneously hypertensive rats (SHR)<sup>231</sup>, transgenic HER-2/neu mice<sup>40</sup> and increase longevity and reduce tumourigenesis in female SHR mice except when given at an old age when it led to reduced life-span<sup>232</sup>.

Although telomere shortening is seen as being involved in aging and carcinogenesis<sup>233</sup> maintenance of telomere length via telomerase expression is vital for endometrial cancer cells to remain proliferative<sup>234</sup>. Metformin may inhibit telomerase activity by inhibiting the hTERT gene expression which encodes the catalytic subunit of telomerase in these cells<sup>234</sup> and by decreasing telomerase activity in the colon cancer cell line SW -480<sup>235</sup>.

## 10. Inhibition of Cancer stages

The tumour suppressor protein p53 regulates the cell cycle (Fig 1) and by cell cycle arrest or apoptotic initiation guards against all stages of cancer including: initiation due to DNA damage; abnormal proliferation; telomere erosion; hypoxia/angiogenesis; loss of support/survival factors; and chemoresistance<sup>236</sup>. Metformin via AMPK activates p53 directly and/or by inhibiting AKT and MDM2 (Fig 1) (see above) and has been found to selectively inhibit p53-deficient colon cancer cell growth and induce autophagy<sup>8</sup>. The p28<sup>GANK</sup> oncogene (overexpressed in many cancers) was shown to inhibit p53 and activate the PI3K/AKT/HIF -1a pathway whereas inhibition of the pathway attenuated p28<sup>GANK</sup> - mediated epithelial-mesenchymal transition (EMT) and migration<sup>237</sup>.

Metformin is known to inhibit NF-κB (see above). A review by Nishikori (2005)<sup>238</sup> on lymphoid malignancies details how NF-κB may contribute to these malignancies and how NF-κB transcribes genes that facilitate tumour progression: inflammation<sup>35;238</sup> (TNF, IL-1, chemokines), cellular immortality (telomerase), cell survival (BCL-X<sub>L</sub>, cIAP, XIAP, cFLIP), angiogenesis (VEGF, TNF, IL-1, IL-8), proliferation (TNF, IL-1, IL-6, cyclin D1, cMYC), tumour promotion (COX2, iNOS, MMP-9, uPA) and metastasis (ICAM-1, VCAM-1, ELAM-1)<sup>238</sup> (see Nishikori 2005<sup>238</sup> for abbreviations). Recent studies, however, have shown that there is a complex relationship between NF-κB and lung metastasis *in vivo*

mammary tumour models and that for a brief initial time period NF- $\kappa$ B activation in macrophages may lead to anti-metastatic effects<sup>239</sup> complex opposing effects of NF- $\kappa$ B have also been demonstrated for other cancers<sup>240;241</sup>. Nevertheless, NF- $\kappa$ B inhibition is one mechanism by which metformin may inhibit different cancer stages.

#### a. Initiation

Results from observational studies demonstrate metformin's cancer-preventing properties (see above). *In vivo* studies such as the prevention of tobacco-induced lung cancer in mice also demonstrate metformin's efficacy<sup>7</sup> The molecular reasons for this are shown in the various pathways studied mainly *in vitro* (Figs 1-4).

#### b. Tumorigenesis

Survivin is an IAP (inhibitor of apoptosis protein) important in tumourigenesis and is regulated by p53<sup>242</sup>. Survivin is not expressed in non-proliferating adult tissues but is highly expressed in cancer<sup>243;244</sup> Different variants exist and possibly have a role in different stages of cancer development<sup>243</sup>, survivin-2B being important in tumour progression<sup>245</sup>. Survivin may inhibit caspase activity, acts at the mitotic spindle checkpoint<sup>246</sup> to promote mitosis and promotes chondrosarcoma chemoresistance *in vitro*<sup>244</sup>.

In lung cancer carcinogenesis *in vitro* and *in vivo* studies, deguelin-induced AMPK activation was shown to inhibit survivin expression by inhibiting p70 S6K via inhibition of the AKT-mTOR1 pathway which then induces apoptosis<sup>247</sup>. Metformin would be expected to act similarly and was found to have a comparable effect in *in vitro* breast cancer studies in an AMPK dependent and/or independent manner<sup>248</sup> (Fig 4).

#### c. Angiogenesis

Induction of HIF-1 via the Ras pathway (see above) and activation and stabilization of HIF-1 $\alpha$  through hypoxia in solid tumours results in the transcription of VEGF- $\alpha$  and Ang-2 which initiate angiogenesis<sup>145</sup>. Moreover, ET-1 activates the PI3K-AKT-mTOR pathway (Fig 1) causing inhibition of PHD2 (Fig 4) promoting HIF-1 $\alpha$  stability leading to angiogenesis and melanoma cell division<sup>169</sup>. Metformin, via AMPK, was found to inhibit insulin and IGF-1 induced HIF-1 $\alpha$  in retinal epithelial ARPE-19 cells<sup>167</sup> and also inflammatory angiogenesis in murine sponge implants<sup>249</sup>. Angiogenesis via NF- $\kappa$ B and ERK pathways was shown to be decreased by metformin's activation of thrombospondin-1 in women with PCOS<sup>250</sup>. In contrast, metformin was reported as inducing an angiogenic phenotype using MDA-MB-435 cells injected into mice<sup>251</sup> however, these cells are most likely to be M14 melanoma cells and cannot be used as a breast cancer model<sup>252;253</sup> although it may indicate an angiogenic effect of metformin on some melanoma cells. Moreover, metformin was found to powerfully stimulate proliferation in one melanoma cell line (nRASQ61K)<sup>254</sup>.

#### d. Invasion

HIF induces several genes involved in invasion. These include those governing proteolysis of surrounding areas, cell migration and adhesion<sup>145</sup>. Hypoxia, thus HIF, induces multi-drug resistance (MDR) and glycolysis in cancer cells<sup>255</sup> (see below).

##### i. Proteolysis

Matrix metalloproteinases (MMPs) are zinc-containing endopeptidases responsible for extracellular matrix (ECM) proteolysis particularly collagen and fibronectin and are involved in cancer metastasis and angiogenesis<sup>256</sup>. Tumour-secreted MMPs destroy ECM in surrounding tissues allowing invasion of the

blood vessel basement membrane and metastasis to remote organs. PI3K and MAPK (mitogen activated protein kinase) pathways contribute to MMP-9 expression along with transcription factors AP-1 (transcription factor activator protein-1), NF- $\kappa$ B and SP-1 (specificity protein -1). Metformin was found to suppress MMP-9 activation by blocking Ca<sup>2+</sup> influx and the PKCa/ERK and JNK/AP-1 pathways inhibiting human fibrosarcoma cell migration, invasion and potentially metastasis<sup>256</sup>.

## ii. Migration

HIF can induce transcription of many genes including those controlling energy metabolism, survival, pH and cell migration<sup>145</sup>. Vascular cell migration is controlled by VEGF-A when sprouting neovessels move into hypoxic tumour areas<sup>145</sup> by inducing external vessel sprouting and promoting migration into the tumour<sup>257</sup>. Endothelial cells at the sprout tips use long filipodia full of VEGFR-2 (VEGF receptor-2) to navigate and migrate<sup>145</sup>. This “angiogenic switch” is needed for invasion into surrounding tissue and to metastasize to remote sites<sup>257</sup>. “Vasculogenic mimicry” is thought to enable tumour cells to acquire endothelial cell features and line the vessels<sup>258;259</sup> and one model suggests that in glioblastoma these cells may be differentiated from tumour stem-like cells<sup>259;260</sup>

Mutations in PTEN lead to enhanced cell migration. It was found that PTEN-deficient cell migration was governed by the PDK1-AKT pathway in mouse fibroblasts (MEFs)<sup>261</sup> and in glioma cells<sup>262</sup>. Metformin was shown to suppress tumourigenesis in PTEN-deficient mice (see above)<sup>124</sup> and migration in glioma cells<sup>262</sup>.

Other cell-migratory genes include PGI/AMF (phosphoglucose isomerase/autocrine-motility factor), the spreading factor (cMET) and transforming growth factor TGF- $\alpha$ . The latter is involved in EMT that is responsible for downregulation of epithelial cadherin (E-cadherin) which is essential for cell-cell adhesion. HIF induces LOX (lysyl oxidase) in hypoxic conditions which transcribes SNAI1, an E-cadherin inhibitory gene<sup>145</sup>.

## iii. Stem cells

A recent review<sup>263</sup> proposes the concept that a normal cell mutates to become a cancer-initiating cell and is not necessarily a cancer stem cell (CSC) which is a subset within the tumour and which sustains or propagates tumour growth<sup>263</sup>. Cancer stem cells or side population (SP)<sup>264</sup> have been defined as cells with self-renewal capacity and the ability to produce multiple distinct differential cell types to form all cell types that are found in the mature tissue<sup>265</sup>. CSCs have been identified in leukaemia<sup>266</sup>, melanoma<sup>267</sup>, multiple myeloma<sup>268</sup> and brain<sup>269</sup>, breast<sup>270</sup>, colon<sup>271</sup>, ovarian<sup>272</sup>, pancreatic<sup>273</sup>, prostate<sup>274</sup> and endometrial<sup>275</sup> cancers.

Cell surface adhesion molecules can be used as markers for CSCs<sup>269</sup>. Differential expression of one or more of these markers indicates that tumour-initiating cell heterogeneity exists for different tumour types<sup>268;272-276</sup>. For instance, CD133 (human Prominin-1, AC133) has been found in brain, prostate, colon, ovarian and endometrial CSCs<sup>275</sup>. CD133<sup>+</sup> cells have demonstrated increased *in vivo* tumour initiation, asymmetric cell division and increased chemotherapeutic resistance compared with CD133<sup>-</sup> cells. CD133 expression has been shown to be epigenetically controlled in normal endometrial and cancer cells<sup>275</sup> and also in ovarian cancer cells<sup>277</sup> where demethylation produces increased expression. CD34<sup>+</sup> is an important marker in chronic myeloid leukaemia stem cells where hypermethylation deactivates HOX genes<sup>278</sup> and CD271<sup>+</sup> indicates melanoma stem cells<sup>267</sup>.

The CD44<sup>hi</sup>/CD24<sup>lo</sup> phenotype indicates breast cancer stem cells together with EMT traits<sup>279</sup> whereas CD44<sup>hi</sup>/CD24<sup>hi</sup> indicates shorter distant metastasis-free survival (DMFS) in MDA-MB-468 triple negative breast cancer patients<sup>280</sup>. Failure of HER-2 overexpressing breast carcinoma cells to respond to the anti-HER-2 monoclonal antibody Trastuzumab is caused by Trastuzumab resistant/CD44 over-expressing

tumour initiating stem cells<sup>281</sup>. Metformin was found to act synergistically with Trastuzumab to suppress self-renewal and proliferation of HER-2 positive CSC/progenitor breast carcinoma cells<sup>281</sup>.

It has been purported that CSCs and non-stem cancer cells (NSCCs), although having different microRNA and mRNA profiles, are converted into each other in both directions in dynamic equilibrium<sup>282;283</sup> where about 10% of the cellular population have been shown to be CSCs in a breast oncogenesis model<sup>282</sup>. CSCs appear to be a more extreme version of transformed cells than NSCCs and have a stronger inflammatory feedback loop involving high NF- $\kappa$ B and Lin28, low Let-7 and high IL-6 (see above and Fig 3). It was found that in breast and prostate cancer cell lines, CSC-secreted IL-6 (and possibly other molecules) converts some NSCCs into CSCs through the inflammatory feedback loop. The rate of CSC formation depends on their proportion of the cellular population, the amount of IL-6 secreted, the IL-6 receptor level and the response of the NSCCs to IL-6 concentration<sup>282</sup>. It was suggested that conventional therapy should be combined with metformin which selectively kills CSCs<sup>282;284</sup>.

Like somatic stem cells, CSCs are reported to be relatively inactive compared with more differentiated tumour cells and having a slow cycling rate demonstrate defense against drug therapies which target the fastest dividing cancer cells<sup>285</sup> however the SP population in the MHCC-97L HCC cell line showed similar growth patterns to non-SP cells<sup>283</sup>. This may be because SP phenotype and "stemness" may vary between different cancer cells tissues with different ABCG2 membrane transporter expression<sup>283</sup> and may result in different responses to different therapies.

The function of membrane transporters belonging to the ATP-binding cassette (ABC) membrane transporter class is to efflux toxic compounds from cells. These transporters include ABCB1, ABCC1 and ABCG2 and they mediate most human multidrug resistance<sup>285</sup>. As with cell surface adhesion molecules, epigenetic regulation of membrane transporters takes place and may be downregulated in chemoresistant cells<sup>285</sup>.

ABCG2 (also known as BCRP1 – breast cancer resistance protein 1) correlates with cancer-stem-like phenotypes and is an important MDR mediator<sup>285;286</sup>. The PTEN and/or the PI3K/AKT pathway regulates ABCG2<sup>287</sup> activity and inhibition of the PI3K/AKT pathway resulted in decreased ABCG2 activity<sup>287</sup> in glioma endothelial stem-like cells<sup>264</sup> in HCC cells<sup>283</sup> and *in vivo* bone marrow cells<sup>288</sup>. mTOR did not take part in this pathway and loss of PTEN increased glioma endothelial stem cell population<sup>264</sup>. RTK (receptor tyrosine kinase) growth factor membrane receptors such as EGFR activate the AKT pathway which induces ABCG2 activation and efflux activity whereas inhibition of these receptors by tyrosine kinase inhibitors will prevent this activity<sup>285</sup>. Metformin's effects on the AKT pathway may explain its inhibitory effects on CSCs. However, inhibition of ABCG2 could result in neurotoxic effects as this membrane transporter helps maintain the blood-brain barrier (BBB)<sup>285</sup>. Orally administered metformin may cross the BBB because it has been found in the cerebrospinal fluid of treated diabetic rats<sup>289</sup>.

ABCG2 has been found to reduce intracellular concentrations of chemotherapeutic agents such as Doxorubicin and Mitoxantrone<sup>285;290</sup>. Nevertheless, metformin and Doxorubicin acted synergistically to kill both CSC and NSCC breast cancer cells in culture<sup>284</sup>. This combination therapy also reduced tumour mass and prevented relapse more than either drug alone in xenograft mouse models which remained tumour-free for at least two months after the therapy ended. Metformin alone was found to inhibit cellular transformation and selectively kill CSCs in 4 genetically different types of breast cancer<sup>284</sup>.

#### iv. Epithelial-mesenchymal transition (EMT) and Metastasis

EMT induction in cancer cells is proposed to effect invasion and metastasis<sup>291</sup>. A review<sup>292</sup> looking at pancreatic cancer proposed that several cytokines and growth factors induce EMT e.g. TGF- $\beta$  &  $\alpha$  and the WNT, Notch and hedgehog pathways and transcription factors SNAI1 and ZEB1. This leads to loss of E-cadherin and cytokeratin and conversion of the epithelial sheet-like structure to the mesenchymal single cell spindle-like morphology with increasing vimentin, fibronectin and N-cadherin. The latter cells can become desmoplastic, invasive, stem-like and demonstrate chemoresistance<sup>292</sup>. MicroRNAs act epigenetically to mediate TGF- $\beta$ -induced EMT<sup>291</sup>. Furthermore, TGF- $\beta$ -stimulated EMT suppressed murine mammary epithelial cancer cell branching induced by EGF/EGFR and generated large dense hyperinvasive spheroids in response to EGF<sup>293</sup>.

CSCs demonstrate EMT markers such as increased secretion of TGF- $\beta$ <sup>294</sup>. Moreover, ET-1 has been shown to confer acquisition of chemoresistance and EMT phenotype in epithelial ovarian cancer (EOC) cells and to activate AKT and MAPK pathways<sup>295</sup>. TGF- $\beta$  activates SNAI1 which acts upstream of PI3K to induce EMT in lens epithelial cells<sup>296</sup> whereas TWIST activates  $\beta$ -catenin and AKT pathways to maintain EMT in MCF breast cancer and HeLa stem-like cells<sup>297</sup> and stem-like characteristics<sup>297</sup>. Thrombin has been found to be associated with deep vein thrombosis and metastatic cancer (Trousseau's syndrome) through activation of HIF-1 $\alpha$ -induced TWIST<sup>298</sup>. Metformin has been shown to repress EMT genes SNAI1, TGF- $\beta$  ZEB1, TWIST1<sup>294</sup> and reduce  $\beta$ -catenin protein in human osteoblastic Saos-2 cells<sup>299</sup> which may be a reason for metformin's anti-cancer stem cell and anti-fibrotic properties<sup>294</sup>. Metformin also inhibits TGF- $\beta$ -induced loss of E-cadherin in MCF breast cancer cells and cell scattering and accumulation of the mesenchymal marker vimentin in MDCK (Madin-Derby Canine Kidney) cells<sup>294</sup>.

The double-strand break repair protein NBS1 (Nijmegen breakage syndrome 1) overexpression was associated with metastasis of HNSCC (head and neck squamous cell carcinoma) and induction of EMT through upregulation of SNAI1 and its downstream target MMP-2. *In vitro* EMT phenotypes and increased migration/invasion were reversed by siRNA-mediated repression of SNAI1 or a PI3K inhibitor<sup>300</sup>. As metformin inhibits PI3K through AMPK it would be expected to act similarly. Conversely, AMPK activation was shown to inhibit anoikis (cell death after detachment) in transforming MEF cells possibly through mTORC1 inhibition, thus protein synthesis inhibition and bioenergetic conservation<sup>301</sup>.

Recently, the hypothesis that EMT effects metastasis and invasion has been challenged because although EMT has been observed *in vitro* and in animal models it has not yet been observed in human tissue sections<sup>302</sup>. Nevertheless, EMT markers have been found in circulating tumour cells in the peripheral blood of metastatic breast cancer patients<sup>303</sup>.

Nevertheless, Metformin may exhibit anti-invasive and anti-metastatic properties in human endometrial carcinoma (ECC-1) cells<sup>304</sup> because sera from women with PCOS treated for 6 months with metformin inhibited *in vitro* ECC-1 invasion. This appeared to be associated with NF- $\kappa$ B, MMP2/9, AKT and ERK1/2 pathways<sup>304</sup>. Moreover, metformin was shown to suppress the metastasis-associated protein CD24 in MDA-MB-468 triple negative breast cancer cells<sup>280</sup>.

#### v. The "Warburg Effect"

Cancer cells appear to have a specific way of metabolizing glucose which is described as "aerobic glycolysis" also known as the Warburg Effect and resulting in hyperglycolytic rates. ATP is gained through glycolysis with the production of lactate even in aerobic conditions rather than by oxidative phosphorylation. There have been many attempts at explaining the reasons for this<sup>305-312</sup> which has led to

suggestions that cancer may have a self-perpetuating purpose and a competitive advantage over “normal” cells. There have been many reasons given for this, for instance, that the Warburg effect may contribute to initiation of cancer<sup>305</sup> by enhanced glycolysis and decreased respiration which suppresses apoptosis<sup>310</sup>. Moreover, AKT<sup>307</sup> and hexokinase-2 (HK2)<sup>312</sup> (which catalyses the first step in glycolysis) have both been proposed as instigators of the Warburg Effect. AKT activation increases glucose transportation and stimulates HK2 which has a higher than 100fold affinity for glucose than HK4<sup>12;312</sup> and also binds to the mitochondrial membrane accessing mitochondrial ATP for increased glucose phosphorylation<sup>312</sup> leading to high glycolytic rates. Mitochondrial biogenesis can then result in increased ROS production thus mtDNA mutations and damaged mitochondria. HIF-1 production inhibits the conversion of pyruvate to acetyl CoA via PDH (pyruvate dehydrogenase) inhibition, resulting in the shunting of pyruvate to lactate<sup>309</sup>. Another explanation purports that mtDNA mutations and hypoxic conditions cause defective mitochondrial respiration leading to cancer-cell dependency on glycolysis for ATP production. Increased NADH competes with NADPH leading to the inactivation of PTEN and activation of AKT leading to proliferation and cell survival<sup>306</sup>. A straightforward explanation is that TCA products from oxidative phosphorylation and late-stage glycolysis in proliferating tissue are diverted for use in amino acid, lipid and nucleotide production so that glycolysis becomes the main method of ATP production<sup>308</sup> which is pronounced in cancer because of the oncogenes and pathways mentioned above.

As metformin mainly acts through inhibition of the electron transfer chain it would be expected to be ineffective during aerobic glycolysis but metformin is pleiotropic and may use several other pathways (Fig 1) for instance, its proposed inhibition of AMPD<sup>120</sup>. One model proposes the “Reverse Warburg Effect”<sup>129;313-315</sup> which may explain one way that metformin could overcome this problem. In breast cancer, mitochondrial and oxidative damage caused by loss of Cav-1 in stromal fibroblasts may cause increased pyruvate and lactate production during aerobic glycolysis. These products can then be used by adjacent epithelial cancer cells in oxidative phosphorylation in the proposed “Reverse Warburg Effect”<sup>129;313-315</sup>. Metformin was shown to upregulate Cav-1 in co-cultured fibroblasts possibly because of metformin’s blocking of ROS production in mitochondria<sup>129</sup>. mTORC1 signalling was found to be more sensitive to metformin as mouse embryonic fibroblasts (MEFs) became more confluent and increased levels of HK2 localized to the mitochondrial membrane making the cell more sensitive to respiratory inhibitors<sup>12</sup>. Additionally, reassessment of data shows that even with aerobic glycolysis, oxidative phosphorylation does not stop or may even increase in many cancer cells<sup>316</sup>.

## 11. Clinical Application

### a. Different types of cancer

The mechanisms leading to different cancer types are diverse and therefore require different ways to combat them. Different cancers are affected by distinct types and patterns of genetic mutation<sup>317</sup>. Data from observational studies are varied (see above) but show that metformin is apparently more successful against some cancers than others and is sometimes ineffective against some<sup>4;318</sup>. There are possibly several reasons for this, for instance, Cav-1 is a tumour suppressor in some instances (see above) e.g. breast cancer, but a tumour promoter in others (see above) e.g. prostate cancer. One possible cause of efficacy differences could be that metformin has been shown to upregulate Cav-1<sup>129</sup>. Moreover, other genetic variations such as in the *ATM* gene are known to affect patient response<sup>319;320</sup> (see below).

It could be argued that metformin lowers cancer risk because it ameliorates diabetes, a cause of many cancers<sup>321</sup> however, there is an inverse relationship between diabetes and prostate cancer<sup>321</sup> and as

metformin lowers the risk of prostate cancer (refs) there may be other mechanisms that do not involve the insulin receptor pathway etc. (mentioned above) which are amongst metformin's pleiotropic effects.

Metformin was shown to act additively with tamoxifen to reduce breast cancer growth *in vitro*<sup>163</sup> and synergistically with doxorubicin to reduce tumour size and relapse in xenograft mice<sup>284</sup> (see above). Moreover, the proportion of more treatable progesterone-positive breast cancers was higher in diabetic women treated with metformin than sulfonylurea<sup>322</sup>. Aromatase complex activation in BRCA1 mutation carriers (an inactivating tumour suppressor mutation) was contemporaneous with increased oestrogen metabolism into catecholestrogens and their inactivation by methoxylation and metformin may affect both these pathways<sup>323</sup>. Metformin suppressed HER2 oncoprotein overexpression in breast cancer cells<sup>164</sup> (see above) and inhibited HER2 transplantable mammary carcinoma in mice<sup>324</sup>. The drug, via AMPK, was demonstrated to inhibit TN breast cancer *in vitro* by reducing EGFR, MAPK, Src, cyclin D and cyclin E and to reduce tumour growth *in vivo* xenografts<sup>6</sup> and to be an effective neoadjuvant in early breast cancer diabetic patients<sup>325</sup>. Metformin was shown to inhibit the growth of oestrogen receptor-positive human breast carcinoma cells more successfully than those without an oestrogen receptor<sup>326</sup>.

Results for metformin's effects on melanoma have been varied<sup>327;254</sup>. Some *in vitro* studies have shown pro-proliferative effects<sup>254</sup>. However, a recent study<sup>328</sup> demonstrated that the drug blocked proliferation in both an AMPK dependent and independent manner in melanoma cells while leaving normal melanocytes intact. This appeared to happen through cell cycle arrest in the G0/G1 phase, then autophagy and apoptosis. These results were supported through tumour shrinkage in mice<sup>328</sup>.

For most cancers there is a significant contribution from mutations in the RTK-Ras-MAPK pathway (Figs 1 and 2) except prostate<sup>317</sup> which may explain its differences from other cancers. Moreover, metformin was shown to act independently of AMPK in downregulating cyclin D1 *in vitro* and in mice xenograft prostate cancer models<sup>178</sup> and to have no effect on biochemical recurrence after radical prostatectomy in prostate cancer patients<sup>329</sup>.

Metformin, via the inhibition by AMPK of the mTOR pathway, was demonstrated to suppress epithelial cell proliferation thus aberrant crypt foci (ACF) (putative pre-neoplastic lesions) formation in the colorectum of mouse models<sup>330</sup> and intestinal polyp growth in mice with a mutated *APC* tumour suppressor gene<sup>331</sup>. Furthermore, metformin was demonstrated to reverse progesterone resistance in endometrial cancer Ishikawa cells by downregulating glyoxylase 1 (Glo1), a chemotherapy resistance mediator, while simultaneously inhibiting mTOR phosphorylation leading to apoptosis and attenuation of cellular proliferation in progestin-resistant cells<sup>332</sup>.

#### **b. Inhibition of multi-drug resistance**

Metformin was found (via AMPK) to inhibit the multidrug resistant gene (*MDR1*)-mediated P-glycoprotein (P-gp)-dependent drug efflux from MCF-7 multidrug resistant breast cancer cells. Proposed mechanisms were inhibition of the AKT/NF- $\kappa$ B pathway leading to attenuation of NF- $\kappa$ B which is known to be a multidrug resistance mediator and CREB downregulation<sup>333</sup> (also see above).

#### **c. Response to Metformin**

The treatment success of metformin is significantly affected by the tumour suppressor gene *ATM*<sup>334</sup> which encodes for a DNA repair and cell cycle regulator protein that is mutated in ataxia telangiectasia, a disease associated with a predisposition to cancer. It is thought that inhibition of *ATM* attenuates metformin's activation of AMPK either directly or indirectly as it is known that *ATM* phosphorylates LKB1, possibly phosphorylates AMPK itself and also other steps in the insulin-signalling pathway<sup>320</sup>. It was found

that double stranded DNA breaks (DSBs) upregulated *ATM*, which in turn activated LKB1, thus downregulating the oncogene *TCL1* (T-cell leukaemia) and inhibited the nuclear localization of mTORC2 that attenuates GC (germinal centre) B-cell proliferation and lymphomagenesis<sup>335</sup>. A similar process was achieved when Ramos human GC B-cells were exposed to metformin which activated AMPK thus LKB1<sup>335</sup>. Similarly, AICAR-induced AMPK activation resulted in mitochondrial biogenesis in an *ATM*-dependent manner<sup>336</sup>.

Metformin is actively transported into cells by an organic cation transporter (OCT)<sup>99</sup>. The OCT1 genotype has been demonstrated to affect metformin pharmacokinetics<sup>337;338</sup>. Seven OCT1 variants have shown reduced response to metformin<sup>338</sup>. In epithelial ovarian cancer cells where there were OCT1 polymorphisms, phenformin was unaffected and may be the drug of choice in some clinical settings<sup>339</sup>. *In vitro* experiments showed that OCT2 genetic variants may influence renal excretion of metformin and possibly lactic acidosis susceptibility<sup>340</sup>.

Metformin was found to induce cell death in a caspase-dependent and -independent fashion in all breast cancer cell lines tested except MDA-MB-231 cells<sup>341</sup>.

#### d. Aneuploidy/genetics/epigenetics

Aneuploidy is a characteristic of cancer and metformin was shown to selectively impair the accumulation of trisomy 13 and 16 cells in cultured MEFs although AICAR was more effective and inhibited more types of trisomic cells<sup>342</sup>.

Different cancers display different patterns of genetic mutation<sup>317</sup> but metformin has a comprehensive effect on genes associated with cell growth. This was illustrated during a genome-wide analysis of human breast cancer cells where metformin was shown to suppress the expression of genes coding for ribosomal protein and macromolecule biosynthesis and many mitosis-related gene families including kinesins, tubulins, auroras, histones<sup>343</sup>.

Epigenetics is defined as mitotically and/or meiotically heritable changes in gene expression that are not accompanied by changes in DNA sequence<sup>344</sup>. These comprise: methylation of cytosine DNA bases; posttranslational histone modifications; DNA nucleosome positioning; and negative gene control by microRNAs (miRNAs)<sup>345-347</sup>. Comprehensive epigenetic changes including all these mechanisms are now accepted, along with genetic changes, as being a feature of cancer, especially miRNA modifications<sup>347</sup>. Changes in miRNA profile can modulate changes in gene expression that characterize early breast cancer<sup>348</sup> and human oesophageal squamous cell carcinoma cell proliferation<sup>349</sup>. Metformin-treated MCF-7 breast cancer cells exhibited upregulated tumour suppressor miRNAs let-7a and miRNA-96 and downregulated oncogenic miRNA-181a preventing EMT-related self renewal<sup>350</sup>. Demethylation of H3K4me2 (Dimethyl lysine 4 histone H3), an epigenetic transcription chromatin mark at gene promoters, by LSD1 (lysine specific demethylase 1) may repress gene expression<sup>351;352</sup>. Novel biguanides have been found to inhibit LSD1 in colon carcinoma cells effecting re-expression of aberrantly-silenced genes<sup>351</sup>.

#### e. Comparison with other drugs

Phenformin is a more effective activator of AMPK and mTORC1-suppressor than metformin in a wider range of tissues *in vivo*<sup>104;353</sup>. Moreover, it is more effective at inhibiting tumourigenesis in PTEN-deficient mice than metformin<sup>124</sup>. However, it was withdrawn from use in patients because of the fatal lactic acidosis it produces<sup>23;24;354</sup> which could limit its use as an anti-cancer medication<sup>353</sup> unless treatment duration and dose could be adjusted<sup>104</sup>.

Rapamycin is an immunosuppressant that inhibits mTORC1 and attenuates translation by the dephosphorylation of 4E-BP1 (Fig 4) and some cellular mRNAs<sup>141</sup>. It appears that metformin's effects on proliferation and apoptosis are greater than those for rapamycin<sup>99</sup>. This may be because negative feedback attenuation due to mTOR activation by rapamycin in the IRS-1 pathway leads to AKT activation whereas AKT is inhibited by metformin's activation of AMPK<sup>99</sup> (see above).

Rapamycin was found to prevent a greater amount of lung cancer tumourigenesis in mice treated with a tobacco carcinogen (90%) than metformin (72%)<sup>7</sup> possibly because of AMPK deficiency in lung tissue. However, because rapamycin is immunosuppressive, paradoxically, it may induce lung tumour development in patients due to reduced lung- and tumour-associated regulatory T-cells<sup>7</sup> making metformin the clinical preference<sup>7</sup>.

Small molecule A769662 binds directly to AMPK at a different site from AMP<sup>355</sup> and activates it more effectively, suppressing mTORC1 in more tissues *in vivo* than metformin<sup>104</sup>. It also inhibits tumourigenesis in PTEN-deficient mice more effectively than metformin<sup>124</sup>.

AICAR is membrane permeable and becomes ZMP (AICAR monophosphate), an AMP mimetic, on entering the cell, binding to the same allosteric site on AMPK as AMP<sup>356</sup>, this direct association makes it a more powerful AMPK activator than metformin<sup>357;358</sup>. Thus AICAR was found to be more effective in stimulating mouse  $\beta$  cells than metformin whose effects were negligible because of differences in effects on membrane potential<sup>357</sup>.

Although metformin does not activate AMPK as strongly as AICAR, it has many AMPK-independent effects<sup>206;359</sup> (see above), for instance, metformin directly downregulated HER2 protein expression in breast cancer cells whereas AICAR did not<sup>164</sup>. Moreover, although both metformin and AICAR decrease ER stress and UPR activation in cardiomyocytes they may act on different AMPK activation pathways<sup>360</sup>. Prolonged AMPK activation by AICAR may lead to liver cell apoptosis<sup>361</sup> an undesirable side effect<sup>358</sup>.

The novel AMPK-activating compound OSU-53 was found to suppress proliferation in triple-negative breast cancer cells (irrespective of LKB1 status) by 3 to 4 orders of magnitude greater than metformin or AICAR which was supported by oral suppression of xenograft tumours *in vivo*<sup>362</sup>. More recently, the metformin derivative HL010183 has been found to be up to 100 times more powerful than metformin in triple negative breast cancer cells and a xenograft model at a lower dose than metformin<sup>363</sup>.

There are at least 6 ways that AMPK can be activated by various compounds<sup>355;355;364</sup> some with great potential but these may take years to develop. It is metformin's pleiotropic actions and its historic tried and tested efficacy and safety that set it apart.

#### **f. Dose needed for cancer suppression**

As the risk of cancer increases with increased insulin dose<sup>365</sup> the dose of metformin needed for cancer suppression needs to be tested on non-diabetic subjects.

A therapeutic metformin dose of 1500 mg/d given for 6 months reduced fasting insulineaemia by an average of 22.4% in early breast cancer patients without overt type 2 diabetes<sup>83</sup>. As an association between preoperative insulineaemia and breast cancer progression rate was reported in a cohort study<sup>366</sup> a phase III randomized trial was suggested to assess metformin's antitumoural efficacy<sup>367</sup>. Patient selection should be based on pharmacogenetic, oestrogen production and signaling aspects and metformin targets within tumours<sup>367</sup>.

PCOS patients were given 500mg of metformin 3 times a day for 30-32 days. A significant reduction in plasma insulin and testosterone and an increase in SHBG concentrations were found<sup>368</sup>.

The equivalent of a therapeutic dose of metformin in high glucose concentration-exposed endothelial cell culture was deemed to be 100µmol/L which prevented mitochondrial PTP opening-related cell death in response to hyperglycaemic conditions<sup>212</sup>.

A phase I trial, performed on 11 patients with various types of advanced solid tumours, showed that the maximum tolerated dose of metformin with temsirolimus was 500mg daily and 20mg weekly, respectively. A patient with head and neck cancer exhibited a partial response, 6 patients were stable and 2 patients' cancer progressed<sup>369</sup>.

Metformin was found to be absorbed by the entire rat intestine but mainly at the duodenum. This was shown to be concentration dependent both by active transport and passively. When extrapolated to humans it was estimated that 74%-90% would be absorbed by the intestine<sup>370</sup>. Greater AMPK activation was achieved with higher concentrations of metformin when applied to cardiomyocytes resulting in reduced UPR and ER stress<sup>360</sup> (see above). Moreover, metformin was demonstrated to decrease human colon cancer cell (SW-480) proliferation in a time- and dose-dependent manner<sup>235</sup>.

The metformin dose usually used in subjects of the observational studies is the standard pharmacological dose of 1500 mg/d (see above). However, it can be seen from other studies that efficacy increases both with dose concentration and temporarily. Therefore, it appears that the maximum-tolerated dose would be most efficacious in preventing/inhibiting cancer. However, in studies where metformin acts synergistically with another agent a much lower dose is needed<sup>284</sup> (see above). A Taiwanese diabetic cohort study found that a metformin dose of 500mg/d was enough to reduce cancer incidence and may represent a threshold dose<sup>371</sup>. Thus, effectiveness depends upon many factors including patient characteristics, genetics and cancer type. There are trials underway to determine the maximum tolerated dose (refs).

#### **g. Resistance to metformin and biomarkers for its efficacy**

The feedback loop whereby mTORC1 and ribosomal protein S6 activation leads to inhibition of IRS1 at Ser<sup>639</sup> thus inhibiting downstream effectors including AKT<sup>99;123</sup> is detailed above (shown in Figure 1 in red). Research has shown that where activation of AMPK by metformin causes inhibition of this feedback loop, it is compensated for by the inhibition of IRS-1 by the phosphorylation of the inhibitory site at Ser<sup>789</sup> which limits downstream signalling<sup>99</sup>. Nevertheless, other research has shown that cells become refractory to the effects of metformin with chronic exposure possibly because of the feedback inhibition<sup>372</sup> thus suggesting that anti-IGF-1R antibodies or small molecule IGF-1R compounds may be used to inhibit activation of IRS-1 pathway<sup>372</sup>. Induction of IGF-1R before and after metformin use may be used to audit its action<sup>372</sup>.

Various biomarkers have been suggested to assess metformin's efficacy, for instance, Cyclin D, other cell cycle and proliferation biomarkers, hormonal levels, IGF pathway markers and p53 levels<sup>43</sup> (see above). Other suggested predictive markers include expression of LKB1 which is an upstream activator of AMPK (Fig 1) and is often lost or downregulated in cancer. Tumour LKB1 levels before and after metformin treatment may indicate its potential and actual treatment success<sup>373</sup> and similarly, mTOR levels may be assessed before and after treatment<sup>373</sup>. Moreover, as metformin has been shown to reduce inflammation thus inflammatory markers which lead to cancer initiation and progression (see above), these marker levels may also be used to assess metformin's effectiveness. Nevertheless, biomarker development requires validation which is methodologically challenging<sup>374</sup>.

## h. Application in non-diabetic subjects

The measurement of patient characteristics including biomarkers mentioned above are needed to assess metformin's efficacy in non-diabetic subjects. For instance, metformin was shown to inhibit injected colon cancer tumour growth and reduce receptor activation in hyperinsulaemic mice independently of LKB1 expression. However, in non-hyperinsulaemic mice only LKB1-negative tumours were inhibited possibly through ATP depletion because of an inactive AMPK pathway<sup>375</sup> thus extrapolation to humans would mean that insulin and LKB1 status would need to be known. The only non-diabetic metformin-treated group of patients is women with PCOS. However, PCOS is associated with insulin resistance and obesity<sup>250</sup>. Moreover, sera from PCOS women increased *in vitro* migration and angiogenesis and showed more NF- $\kappa$ B, ERK 1,2 & 5 phosphorylation when added to HMEC compared with matched normal controls<sup>250</sup> making PCOS women poor "normal" subjects for metformin trials.

### i. Adjuvant effects

Metformin can be used as an adjuvant in chemotherapy but the effects may depend upon the therapy used and the type of cancer. For instance *in vitro* metformin acted synergistically with Trastuzumab to suppress self-renewal and proliferation of HER-2 positive breast carcinoma CSC/progenitor cells<sup>281</sup> (see above). Moreover, when metformin or phenformin were combined with 2DG (2-deoxyglucose) and incubated with colon cancer cells, enhanced growth inhibition and reduced acidification were observed<sup>376</sup>. Metformin and 2DG together had significant anti-tumoral effects in mouse xenograft models through combined respiratory and glycolytic inhibition<sup>377</sup>.

Metformin given in water alone was shown to inhibit ovarian cancer growth, proliferation, angiogenesis and metastasis in mice but acted synergistically with cisplatin to reduce tumour size by approximately 90%<sup>378</sup>. Combination of metformin and cisplatin *in vitro* resulted in synergistic action in epithelial ovarian cancer cell lines to induce apoptosis<sup>9;196</sup> and also in mouse B16 melanoma cells<sup>327</sup>. Synergistic action by metformin was also observed in osteo- and fibrosarcoma cells with cisplatin or doxorubicin<sup>379</sup>, with doxorubicin in CSC and NSCC breast cancer cells in culture<sup>284</sup> and in tumour inhibition in xenograft mouse models<sup>284</sup> (see above). Importantly, metformin acted antagonistically with cisplatin in human<sup>327;380</sup> and rat glioma cell lines<sup>327;380</sup>, mouse fibrosarcoma lines<sup>327;380</sup> and human neuroblastoma and leukaemia cell lines<sup>327</sup>.

Oral administration of metformin to xenografted mice with either doxorubicin, paclitaxol<sup>381</sup>, or carboplatin inhibited tumour growth and prevented relapse of several cancer types<sup>382</sup> and reduced the dose of doxorubicin needed thereby possibly increasing standard chemotherapy effectiveness<sup>382</sup>.

Chemotherapy involves the use of mitotic inhibitors (MI) which also damage normal cells. Using three normal human fibroblasts and epithelial cell types and mutant p53 cancer cells, a combination of rapamycin and metformin was found to potentiate chemotherapy with the MIs paclitaxol and nocodazole while protecting normal cells<sup>383</sup>.

Metformin and Vemurafenib (a BRAF inhibitor) were tested on several melanoma cell lines. It was found that metformin alone inhibited proliferation in 12 out of 19 cell lines and synergistic effects of the two drugs were found in many lines with antagonistic effects in some<sup>254</sup>.

### j. Side and other effects

The most common side effect seen in patients using metformin is gastrointestinal upsets<sup>384;385</sup> such as nausea, vomiting, abdominal pain and flatus (around 30%) possibly because of metformin-mediated 5HT (5-hydroxytryptamine) and other neurotransmitter release within the duodenal mucosa<sup>386</sup>. Other side

effects include vitamin B-12 deficiency<sup>387</sup> (see above) and lactic acidosis<sup>354;388</sup> possibly because of respiratory inhibition<sup>354</sup>, pancreatitis<sup>388</sup> and respiratory alkalosis<sup>389</sup> which usually occur in patients with renal failure or another underlying health problem.

A meta-analysis showed that metformin decreased LDL and triglyceride levels in patients<sup>384</sup>. The drug has also been found to inhibit tau expression in Alzheimer's plaques<sup>206</sup> (see above), modulate LH (luteinizing hormone) and the LH/FSH (follicle stimulating hormone) ratio particularly in PCOS patients<sup>390</sup>, lower blood pressure<sup>391</sup> and potentially to ameliorate neuropathic pain<sup>392</sup>.

## 12. Future

### a. In vitro and in vivo work

*In vitro* work is quick and informs molecular pathways but it can only be used as supporting evidence for drug efficacy because cell lines may not act like those in the body and immortalised lines such as HeLa cells develop their own characteristics. Moreover, HeLa cells have cross-contaminated other cell lines<sup>393</sup>. Cells may be wrongly identified, for instance, MDA-MB-435 cell lines used in some breast cancer studies<sup>251;253;394</sup> (see above). Prostate cancer cell lines DU145, PC-3 and LNCaP demonstrate very different characteristics<sup>395-398</sup> and the TSUPR1 cell line may be derived from bladder carcinoma cells<sup>399</sup>.

MCF-7, the most commonly used breast cancer cell line, despite similar morphology, demonstrated different growth rates, hormone receptor content, karyotype and clonogenicity from different laboratories<sup>393</sup> making result comparison difficult. Screening is one option<sup>393</sup>. Nevertheless, cell lines are obtained from more aggressive, often metastatic tumours not primary lesions, making results unrepresentative of the diverse types and stages of cancer<sup>393</sup>. Cell lines do not automatically demonstrate the heterogeneity and the stem-cell dynamics of tumours but may be manipulated<sup>400</sup>. More recently primary tumour cell lines have been established and primary cell cultures have been used as explants and for individual cell culture<sup>393;400</sup>.

*In vivo* investigations are now mainly done through xenografts<sup>400</sup> as direct extrapolations between species are problematic<sup>401</sup>. Early passage cancer cell xenografts correlate well with clinical outcome when combined with mathematical modeling to individualize treatment for patients from whom the tumours are obtained<sup>400</sup>.

Molecular, cell-line, primary or early passage culture and xenograft modeling now optimize pre-clinical experimentation prior to clinical trials. Transplantation using extracellular matrices is now often used in mammary repopulation assays<sup>402</sup>.

### b. Planned trials

### c. Trials on healthy individuals

## 13. Conclusion

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