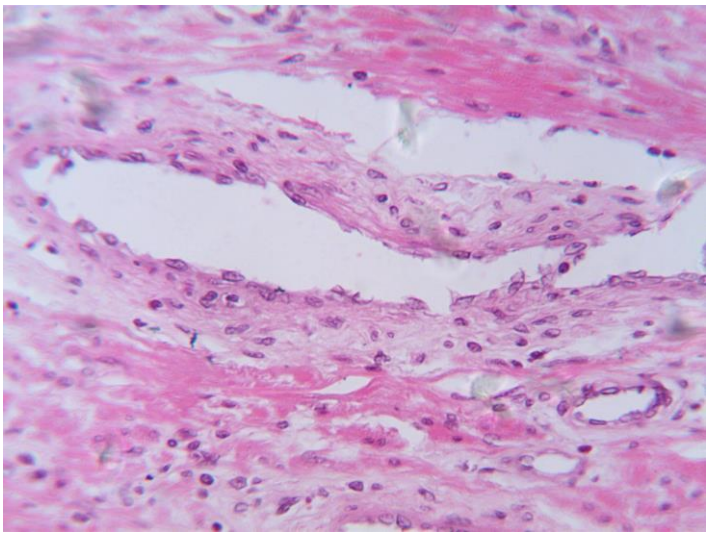

METFORMIN, PROSTATE CANCER, AND EFFICACY

Terrence McGarty

ABSTRACT
This Report discusses the use of metformin in the treatment of prostate cancer. We first examined this topic in 2015 and since then significant additional clinical and scientific insight has been obtained.

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Notice

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1 INTRODUCTION

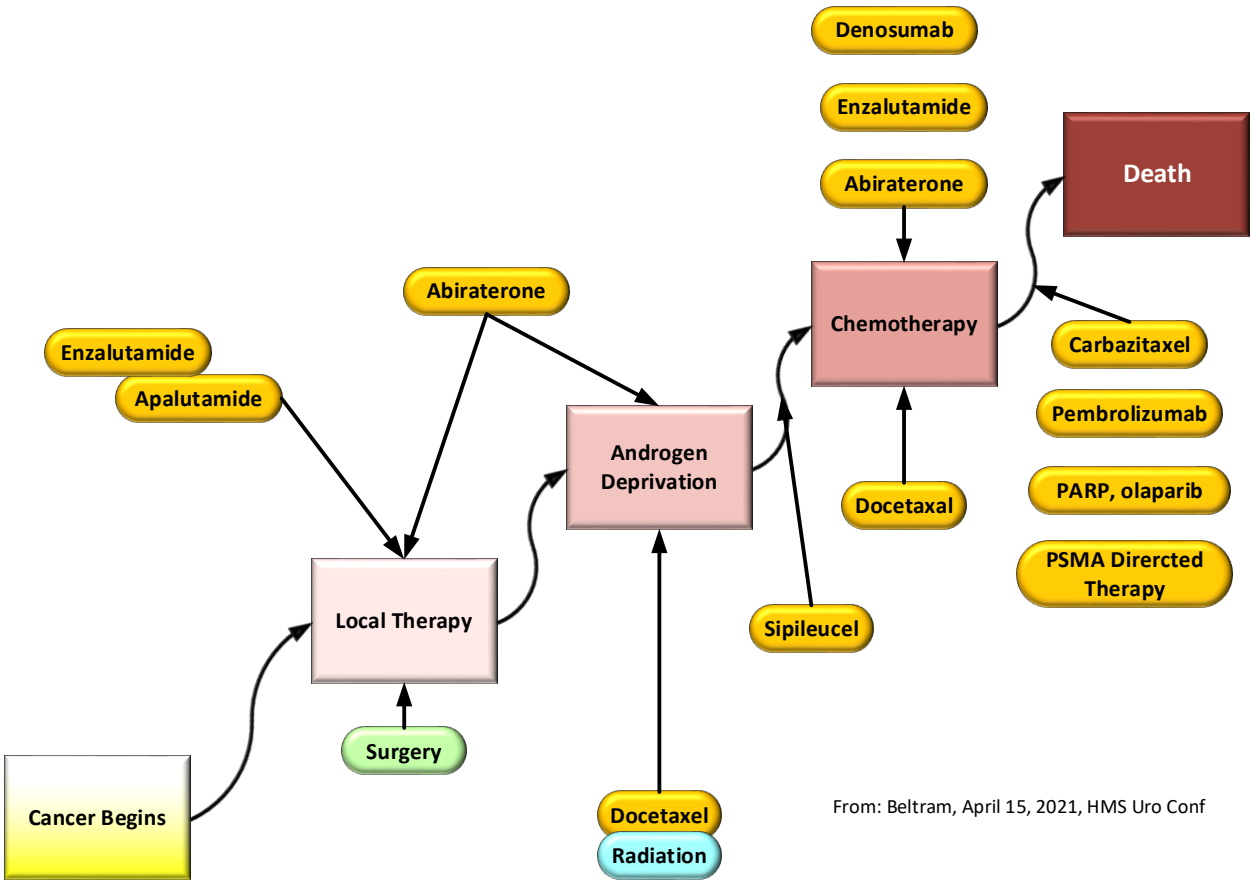
Prostate cancer is a complex disorder and it also very heterogeneous. It is the most significant male cancer with a moderate mortality rate of about 25%. However, this mortality rate is quite confusing since genomic differences, demographic differences and even psychographic differences come into play. Namely there is a small group who have genetic variants which result in an aggressive disease. Second there are demographic groups who defer any testing until it is often too late. The cancer generally is slow growing, unlike ovarian or pancreatic cancer, and thus is properly attended to can be handled with minimal consequence. The problem often is getting the patients tested and followed.

In 2015 we produced a report discussing the research regarding metformin and statins in dealing with prostate cancer (PCa)[1]. This was one of several early reports addressing the use of metformin. In a sense it was an incidental observation. However, in the years since then a significant number of results have been provided further strengthening the utility of metformin before and after a PCa diagnosis.

1.1 CURRENT THERAPEUTICS

There has been a great deal of progress in the past ten years in dealing with PCa. The chart below is from Beltram (as modified) and represents the multiplicity of therapeutics available. This chart presents the current general understanding on how to treat PCa.

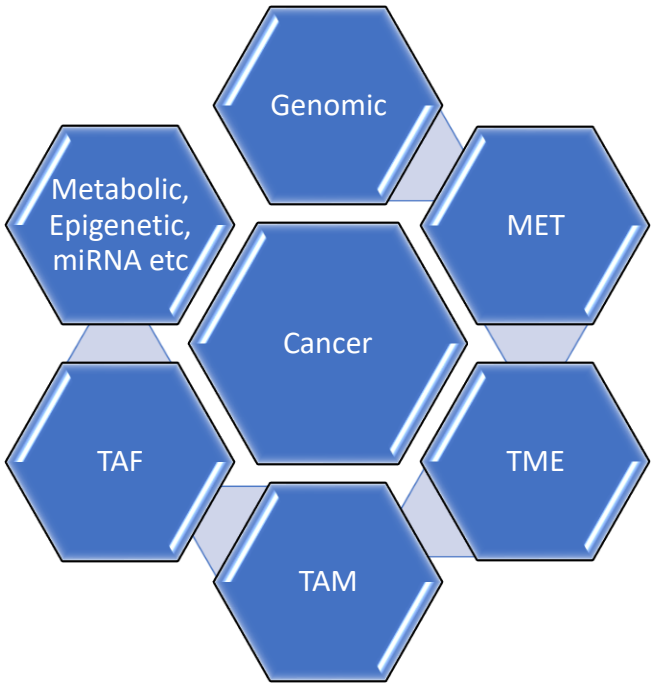
[1] https://www.researchgate.net/publication/351034816_Metformin_and_Statins_in_PCa



As the disease progresses there are tools available to slow and hopefully inhibit the process, even after metastasis and androgen resistance occurs.

1.2 Cancer and Drivers

Cancer has a complicated and intertwined ecosystem that initiates and supports it. It is not just the genetic disruptions alone that engender its overall aggressiveness. We lay out some of these below.



Specifically:

1. Genomic[2]: This is the internal disruption of normal homeostasis in a cell's status. Generally the focus has been on this element and the therapeutic approach has also been focused in disrupting this process.

2. MET, Mesenchymal Epithelial Transition[3]: Cancer cells lose their location specificity and not only proliferate but lose their basic location anchoring. For example, we see melanocytes moving from the basal layer upwards and then downwards. The same happens in PCa with basal and luminal interactions.

3. TME, Tumor micro-environment[4]: The environment surrounding cancer cells often is protective. The TME is a complex of a large set of these protective elements.

4. TAM, Tumor associated macrophages: Macrophages generally are the garbage collectors in the body. M1 macrophages perform a positive task even addressing cancer cells. However, M2 macrophages may exert a protective function for cancer cell clusters.

5. TAF, Tumor associated fibroblasts: We have discussed this at length elsewhere.

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[2] https://www.researchgate.net/publication/343669352_AR-V7_A_Driver_of_Prostate_Metastasis

[3] https://www.researchgate.net/publication/325046881_PCa_mir34_p53_MET_and_Methylation

[4] https://www.researchgate.net/publication/341788660_Fibroblasts_and_Cancer_The_Wound_That_Would_Not_Heal

6. Metabolic[5], Epigenetic[6], miRNAs etc?: Cancer cells have a complex metabolic interaction and this is typified by Warburg processes. In addition, epigenetic issues such as methylation and acetylation have been shown to play a significant role as well. We also have such factors a miRNAs which play a role.

Thus it is essential that when providing a therapeutic we address all of these elements. For example in immunotherapy we generally face two obstacles. First is the TME and affiliated elements blocking cell attack. Second, and this seems to be more critical as it is addressed, that immunotherapy is used as an adjuvant. Namely it is used ex post facto to other treatments, such as surgery or radiation. Recent studies indicate that if used as a neo-adjuvant, namely when tumor burden is higher, it may be significantly better having been trained on a larger tumor set.

1.3 Interest in Metformin

Now for the topic of this note. Metformin is a classic therapeutic for Type 2 Diabetes, T2D. As Zhao et al have noted:

Complex I, AMPK, mTOR, and mGPD have all been suggested as molecular targets mediating the antitumor activities of biguanides. Consistent with Complex I serving as a main target, biguanides preferentially target subpopulations of slower-growing cancer cells that are more reliant on OXPHOS for survival, creating therapeutic opportunities for the combination of biguanides with drugs that act on rapidly cycling populations to exert greater tumor clearance. Targeting compensatory metabolic pathways needed for cancer cells to survive metabolic disruptions may also improve biguanide efficacy in combination.

In addition, biguanides exert effects on the TIME, with the potential to influence the immune recognition and elimination of the tumor. Interestingly, biguanides can also affect host pathophysiology by modulating the gut microbiota, raising the possibility that biguanides may indirectly impact patient response to cancer therapies.

Therefore, it is unknown whether biguanides affect direct targets in cancer cells, the tumor microenvironment, or commensal microbiota, bearing in mind that none of these putative effects are mutually exclusive. Finally, the pharmacodynamic properties of phenformin suggest that it may be preferred over metformin for applications in cancer therapy, as metformin has shown limited efficacy in recent clinical trials possibly due to its more limited pharmacodynamic properties. While the toxicity profile of phenformin is not ideal as a longterm maintenance therapy for type 2 diabetes patients, it is well within the limits for treating cancer.

The incorporation of preclinical and clinical studies of biguanides, including novel derivatives, will help to elucidate biomarkers that predict therapeutic efficacy, define proper patient cohorts

[5] https://www.researchgate.net/publication/322437754_Glucose_Warburg_Cancer_and_Pathways

[6] https://www.researchgate.net/publication/325046881_PCa_mir34_p53_MET_and_Methylation

with sensitivity to biguanides, and guide clinical trials of both mono- and combination therapies in cancer

Whitman et al have also noted:

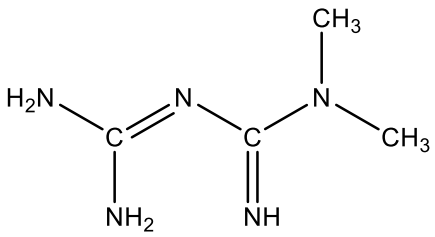
Since epidemiological studies first demonstrated a potential positive effect of metformin in reducing cancer incidence and mortality, there has been an increased interest in not only better understanding metformin's mechanisms of action but also in exploring its potential anti-cancer effects. In this review, we aim to summarise the current evidence exploring a role for metformin in prostate cancer therapy. Preclinical studies have demonstrated a number of antineoplastic biological effects via a range of molecular mechanisms.

Data from retrospective epidemiological studies in prostate cancer has been mixed; however, there are several clinical trials currently underway evaluating metformin's role as an anti-cancer agent. Early studies have shown benefits of metformin to inhibit cancer cell proliferation and improve metabolic syndrome in prostate cancer patients receiving androgen deprivation therapy (ADT). Summary While the body of evidence to support a role for metformin in prostate cancer therapy is rapidly growing, there is still insufficient data from randomised trials, which are currently still ongoing. However, evidence so far suggests metformin could be a useful adjuvant agent, particularly in patients on ADT

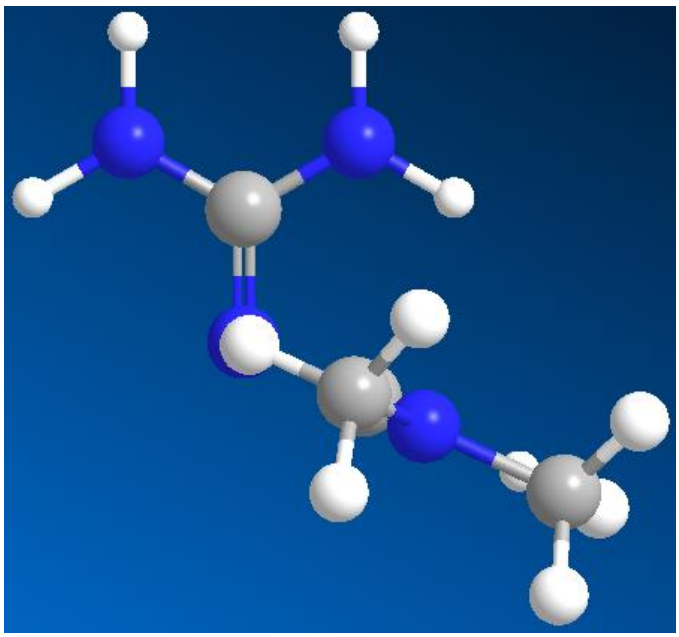
Thus, there appears to be significant potential for the use of metformin as part of an overall therapeutic regime.

2 METFORMIN

Metformin is a classic Type 2 Diabetic control medication and has been used extensively with many patients for several decades. We demonstrate below the areas in which Metformin exercises its influence. Metformin[7] is configured as shown below:

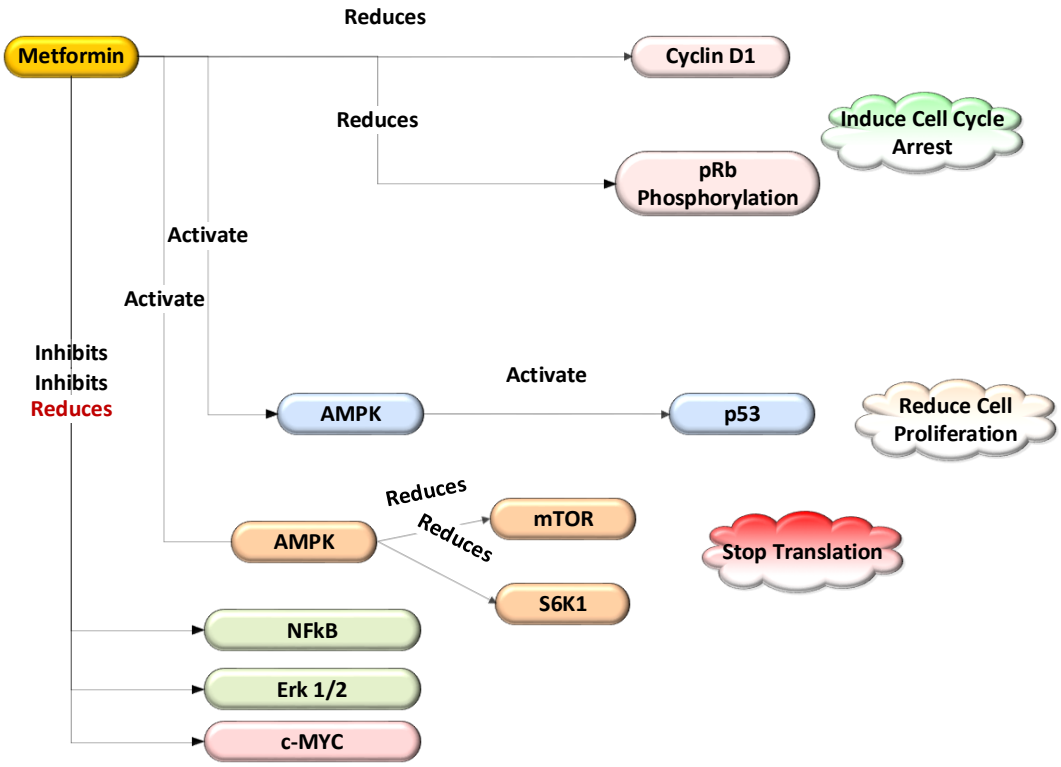


with the 3-dimensional structure as below:

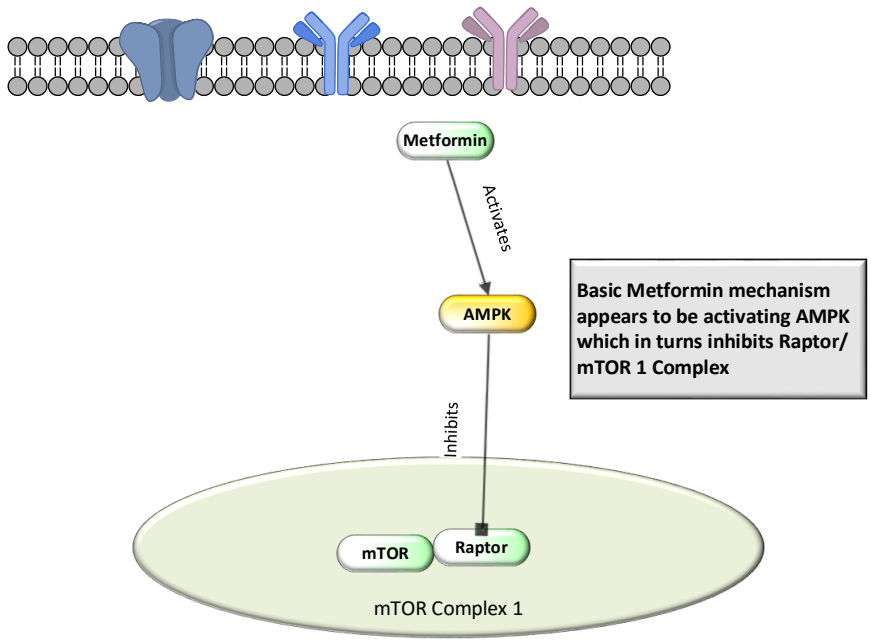


It is a simple molecule but can exert significant impact on multiple metabolic pathways. The impact of metformin on various gene and gene products is shown below

[7] https://pubchem.ncbi.nlm.nih.gov/compound/Metformin



The end state actions shown above clearly show a significant potential for metformin. We shall examine the recent work to date to better understand its potential. The basic step involves the control of AMPK and in turn mTOR. We have examined the latter (mTOR) in some detail before.



Prostate cancer has frequently been seen related to inflammatory processes. The exact connection is yet to be determined. However recent results have indicated that metformin has shown some effect on PCa and a recent paper by Danzig et al shows significant effects with metformin and statins. Both drugs have a certain antiinflammatory role, one in glucose metabolism management and the other through lipid pathways. In this paper we examine both the Danzig et al results as well and the details regarding the specific pathways involved. Specifically, the drugs deal with metabolic related pathways, which is no surprise given the nature of Type 2 Diabetes. However, the statin usage is not directly metabolic but may very well be so.

Shao et al state[8]:

The widely used anti-diabetic drug metformin has been shown to exert strong antineoplastic actions in numerous tumor types, including prostate cancer (PCa).

In this study, we show that BI2536, a specific Plk1 inhibitor, acted synergistically with metformin in inhibiting PCa cell proliferation.

Furthermore, we also provide evidence that Plk1 inhibition makes PCa cells carrying WT p53 much more sensitive to low-dose metformin treatment. Mechanistically, we found that co-treatment with BI2536 and metformin induced p53-dependent apoptosis and further activated the p53/Redd-1 pathway.

Moreover, we also show that BI2536 treatment inhibited metformin-induced glycolysis and glutamine anaplerosis, both of which are survival responses of cells against mitochondrial poisons. Finally, we confirmed the cell-based observations using both cultured cell-derived and patient-derived xenograft studies. Collectively, our findings support another promising therapeutic strategy by combining two well tolerated drugs against PCa proliferation and the progression of androgen-dependent PCa to the castration-resistant stage.

For example, in the work of Margel et al they note:

By using fractional polynomials, we verified that the association between cumulative metformin use after PC diagnosis and PC specific mortality is linear. Onmultivariable analysis, for each additional 6 months of metformin use after PC diagnosis, there was a 24% reduction in PC-specific mortality (adjusted HR [aHR], 0.76; 95% CI, 0.64 to 0.89). Increasing durations of cumulative use of all other antidiabetic medications was not associated with PC-specific mortality.

It reduces, inhibits, and activates a variety of pathway elements all of which control cell cycles and apoptosis. It controls the metabolic cycles that relate to the pathway elements we have shown in the previous sections.

[8] http://www.jbc.org/content/290/4/2024.abstract

The impact of AMPK and in turn p53 is a significant pathway. AMPK is as we have seen a significant metabolic player and metformin modulates it behavior. It manages the Cyclin D1 which controls cell cycle growth. One may wonder why so effectively in the prostate, however. The mTOR management is via AMPK as well and then through mTOR C1.

As Mendelsohn et al state:

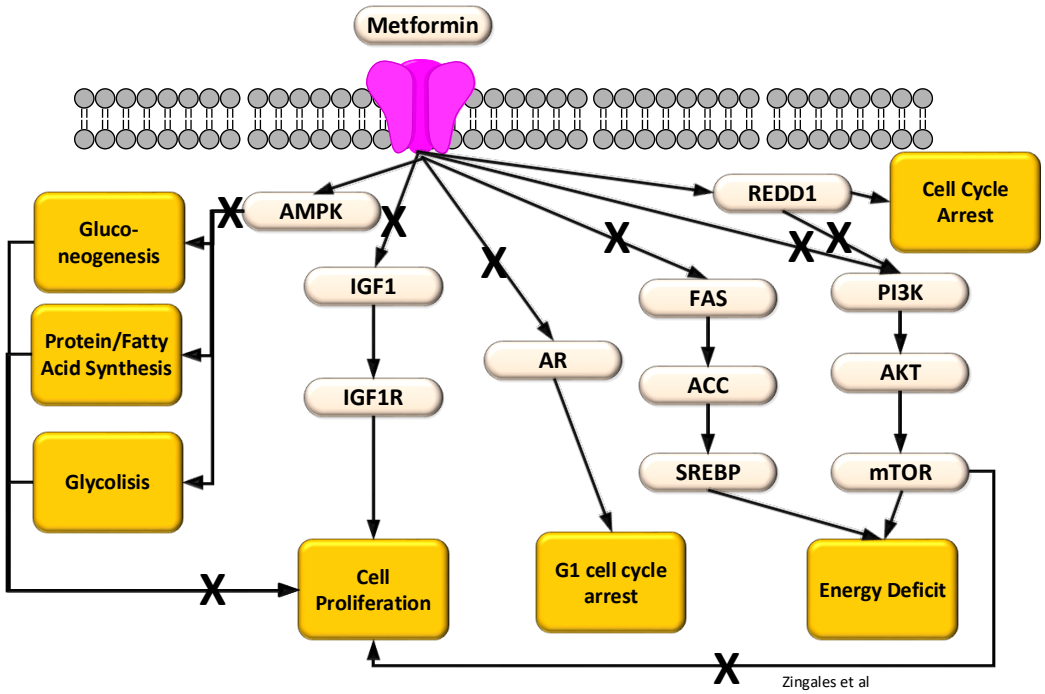
Metformin belongs to the biguanide class of antidiabetic drugs and activates the LKB1/AMPK axis (mediating glucose and energy homeostasis) and inhibits cancer cell viability through the inhibition of mTOR. Metformin can also downregulate mTOR and subsequent cell growth through AMPK-independent mechanisms. A recent study using mouse models of lung cancer to assess the protective effect of metformin suggested two possible mechanisms: decreased levels of circulating insulin and lowered energy stress leading to inhibition of mTOR.

Owing to the fact that studies show metformin is associated with a decreased risk of cancer incidence compared with other treatments (such as insulin) among diabetic patients, metformin is rightfully garnering interest for its role in cancer prevention and therapy and supports further testing in the clinical setting.

The Mendelsohn comment has been demonstrated in Danzig somewhat.

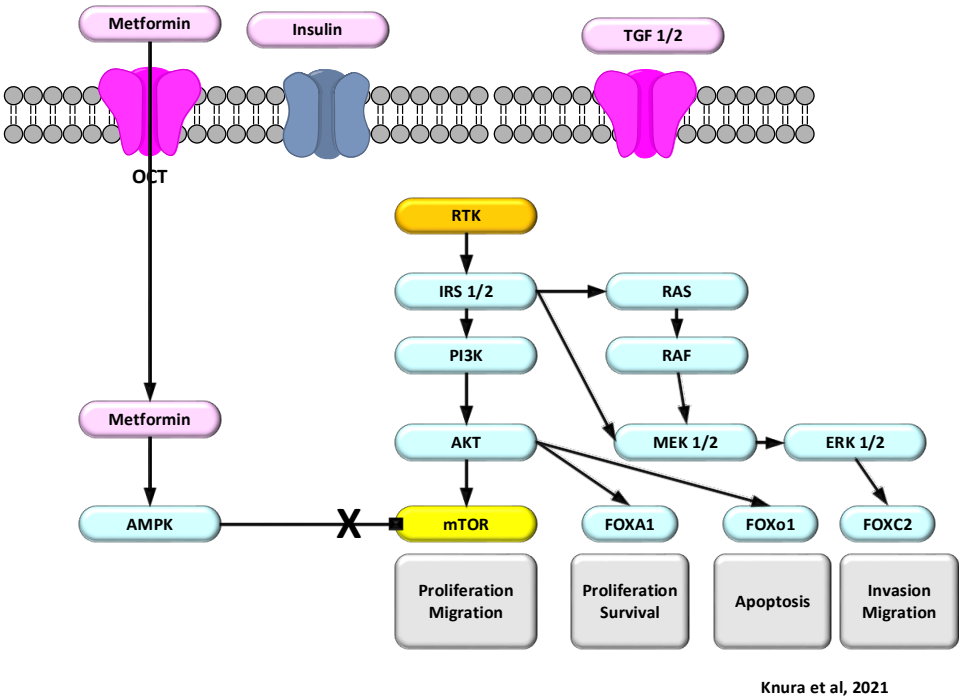
2.1 A PARADIGM

Metformin has been found to be a regulator or controller of a multiplicity of critical cellular pathways, especially those related to malignant progression. We first present a paradigm showing many of these control elements. As Zingales et al have noted:



This is a somewhat complicated and compressed chart but it demonstrates the putative effects of metformin, especially on metabolic actions. AMPK plays a significant role as does mTOR and the REDD1 gene. AMPK is a key element in the control balance of ATP and AMP.

In a similar manner Knura et al note the above in the following manner:



Knura et al follow by noting:

Metformin (Met) is the drug of first choice in type 2 diabetes mellitus. It reduces the level of circulating glucose and is particularly effective against insulin resistance and in obese patients. In the animal models, metformin inhibited proliferation of tumor cells, but not cell migration of PC [60]. Using metformin also induces apoptosis via activation of AMPK (AMP-activated kinase) pathway in prostate cancer cells.

AMPK is a regulator sensitive to cell energy status, it controls the balance between the anabolic and catabolic processes. Through enzyme phosphorylation and regulation of gene expression, it allows cells to adapt to environmental conditions. Inhibiting proliferation is also reached by blocking the cell cycle in G0/G1. Metformin decreases cyclin D1 level, pRb phosphorylation, and increases p27kip protein expression.

Metformin also is effective in lowering IGF-1 and insulin levels. These hormones can stimulate prostate cancer proliferation through activation of the FOXO1 subunit of the androgen receptor. Metformin upregulates REDD1 (regulated in development and DNA response-1) that promotes

cell cycle arrest and inhibits PI3K/AKT/mTOR. These actions lead to tumor suppression and increase apoptosis.

Met also inhibits NF-κB, leading to delay of cell aging. However, modulation of inflammatory cytokines profile leads to improved response against cancer cells [65,66]. Despite the promising outcomes of the wide array of pre-clinical studies, clinical trials considering the risk of PC incidence and progression of this malignancy present with varying results upon administration of Met. The available data present a spectrum of findings of Met having reduction of risk no effect, to even an increased risk of PC.

Similar discrepancy is observed in meta-analyses…statistically significant reduction of PC risk was associated with metformin therapy. These two meta-analyses…are based on older observational studies, and consequently, less patients are included…. Another aspect that should be taken into consideration in clinical studies is the impact of metformin on the progression of disease among patients with already diagnosed PC and further therapy outcomes.

Some previous research … do not support a beneficial correlation between all-cause mortality and metformin use. As well as no association with cancer-specific mortality and metastasis, there is no supporting evidence of a positive impact on the recurrence of PC. In the results of all meta-analyses from the last 5 years, … overall survival among patients with PC treated with metformin was improved. Also, the recurrence of PC among metformin-users in the recent three large meta-analyses is supposed to be decreased. These meta-analyses included a larger patient database than older ones. The mentioned research articles use different survival analysis statistics. The reason for the discrepancy among presented studies could be confounding factors and heterogeneity between research samples.

2.2 AMPK

Cell metabolism is the process whereby a cell uses energy that is made available to it to maintain normal processes and to grow and reproduce as may be required. Normal metabolic processes in a cell allow for the control of all of the elements in a balanced manner. Excess glucose as seen in Type 2 Diabetes can result in quasi-inflammatory states and loss of homeostasis.

Let us focus briefly upon AMPK, AMP kinase, as an initial point to understand the intra-cellular metabolic processes. AMPK is also a key control element in many intracellular pathways[9].

From the paper by Mihaylova and Shaw we have[10]:

One of the central regulators of cellular and organismal metabolism in eukaryotes is AMP-activated protein kinase (AMPK), which is activated when intracellular ATP production decreases.

[9] http://www.cellsignal.com/contents/science-pathway-research-cellular-metabolism/ampk-signaling-pathway/pathways-ampk This is a useful pathway description worth examining in detail.

[10] http://www.nature.com/ncb/journal/v13/n9/full/ncb2329.html

AMPK has critical roles in regulating growth and reprogramming metabolism, and has recently been connected to cellular processes such as autophagy and cell polarity. Here we review a number of recent breakthroughs in the mechanistic understanding of AMPK function, focusing on a number of newly identified downstream effectors of AMPK.

From the work of Shackelford and Shaw we have[11]:

In the past decade, studies of the human tumour suppressor LKB1 have uncovered a novel signalling pathway that links cell metabolism to growth control and cell polarity.

LKB1 encodes a serine–threonine kinase that directly phosphorylates and activates AMPK, a central metabolic sensor. AMPK regulates lipid, cholesterol and glucose metabolism in specialized metabolic tissues, such as liver, muscle and adipose tissue. This function has made AMPK a key therapeutic target in patients with diabetes.

The connection of AMPK with several tumour suppressors suggests that therapeutic manipulation of this pathway using established diabetes drugs warrants further investigation in patients with cancer.

In particular Shackelford and Shaw demonstrate the impact of Metformin on this pathway. As Mendelsohn et al state:

While growth factor–stimulated signaling cascades promote cell growth under favorable conditions, cells have sophisticated nutrient sensing systems that serve to block growth when the internal energy supply is limiting. These regulators ensure that, during periods of intracellular nutrient depletion, metabolites are redirected from anabolic pathways and instead used to fuel catabolic pathways that will provide the energy required to survive the period of nutrient limitation. The AMP-activated protein kinase (AMPK) plays a major role coordinating cellular energy status with appropriate metabolic responses.

AMPK directly senses cellular energy levels in the form of the AMP/ATP ratio. Falling energy levels increase the cellular AMP/ATP ratio, priming AMPK for activation by the liver kinase B1 (LKB1). AMPK phosphorylates multiple targets with the cumulative effect of blocking anabolic reactions and stimulating energy-generating catabolic pathways.

For example, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), with the dual effect of blocking fatty acid synthesis and activating fatty acid oxidation. AMPK also directly inhibits cell growth, both by inducing a p53-dependent cell cycle arrest and by blocking mTOR activity at multiple levels. Through these diverse activities, AMPK functions as a metabolic checkpoint, ensuring that cell growth is halted until bioenergetic conditions are favorable for growth.

[11] http://www.nature.com/nrc/journal/v9/n8/full/nrc2676.html

AMPK is a powerful regulator of cell dynamics. It senses and manages energy via the ATP control cycle. Its impact on p53 which we have discussed earlier is also a major factor which may lead to cell oncogenesis. Thus examining how AMPK reacts to excess glucose and how it can be reset is a key observation.

As Zingales et al note:

Metformin is an insulin-sensitizing oral biguanide used by diabetic patients every day to maintain their glycemic homeostasis. Metformin is an ideal drug: it is well tolerated and inexpensive. Metformin regulates glucose homeostasis exerting an important control of metabolism. In particular, metformin reduces intestinal absorption of glucose and it increases peripheral glucose uptake and its utilization by adipose tissue and skeletal muscles leading to increased insulin sensitivity.

Through AMPK activation, metformin decreases insulin secretion, inhibits gluconeogenesis and energy consuming processes (such as protein and fatty acid synthesis), and stimulates ATP-generating processes (such as glycolysis and fatty acid oxidation). This results in a shift from anabolic to catabolic metabolism and in an inhibition of proliferation*….*

AMPK activation appears the main mechanism through which metformin inhibits cancer growth. AMPK plays a key role in the maintaining of cellular energy homeostasis. It is an important sensor of the AMP/ATP ratio. AMPK appears as a potential anticancer agent when it is highly activated, but it may not be critical as inhibitor of cancer growth when it acts at low levels.

Metformin primarily acts to directly inhibit the mitochondrial respiratory chain which then reduces the production of ATP resulting in an increase in the ratio of AMP to ATP which then results in activation of AMPK. Under energy stress conditions, the tumor suppressor LKB1 (37) is the major kinase involved in the AMPK activation and mTOR reduction. Through the mTOR inhibition, metformin arrests cell cycle and cell growth, because mTOR is a downstream effector of PI3K/ AKT pathway, a signaling pathway linked to cancer cell growth and proliferation. PI3K/AKT/mTOR signaling pathway leads to an abnormal cells proliferation, inhibition of apoptosis, and carcinogenesis.

*…****metformin owns an antiproliferative effect in PCa cells through the activation of pAMPK and subsequent inhibition of downstream mTOR signaling and the induction of cell cycle arrest.*** *In this study, metformin was used in combination with bicalutamide, a known agent used in the hormonal therapy of PCa. It acts blocking the AR and inducing a G1/S phase arrest of the cell cycle. Combining metformin with bicalutamide, the authors obtained a reduction of PCa cell survival, especially in cells expressing functional AR*

The anti-PCa effect of metformin via AMPK activation … demonstrated, in vitro and in vivo, that metformin induces apoptosis and attenuates PCa cell proliferation. Furthermore, a stronger decrease of PCa growth was achieved when metformin was combined with Exenedin-4, a glucagon-like peptide-1 receptor agonists

As Hua et al note:

mTORC1 not only senses growth factors, but also responds to cellular energy. Low cellular energy results in an increase in AMP/ATP ratio, which activates the energy sensor AMP-dependent kinase (AMPK). AMPK stimulates the GAP activity of TSC and then promotes the inhibition of RHEB by TSC, leading to the downregulation of mTORC1. In addition, the TCA cycle metabolite ketoglutarate inhibits mTORC1 through repressing ATP synthase, increasing AMP/ATP ratio and activating AMPK. Cellular energy deficiency usually leads to endoplasmic reticulum stress, which in turn induces the unfolded protein response (UPR). Ire1, ATF6, and PERK are three major mediators of the UPR.

Upon ER stress, ATF6 can induce RHEB expression, which in turn promotes mTORC1 activation and cell survival. However, overactivated mTORC1 is also harmful to cell survival under ER stress. Mutations in TSC1/2 or activation of RHEB renders cells hypersensitive to ER stress-induced apoptosis, which may be due to the downregulation of ATF4/6 by mTOR. Therefore, mTORC1 may have versatile effects on cell survival under ER stress.

2.3 MTOR

We start with a brief overview of mTOR. As NCBI states[12]:

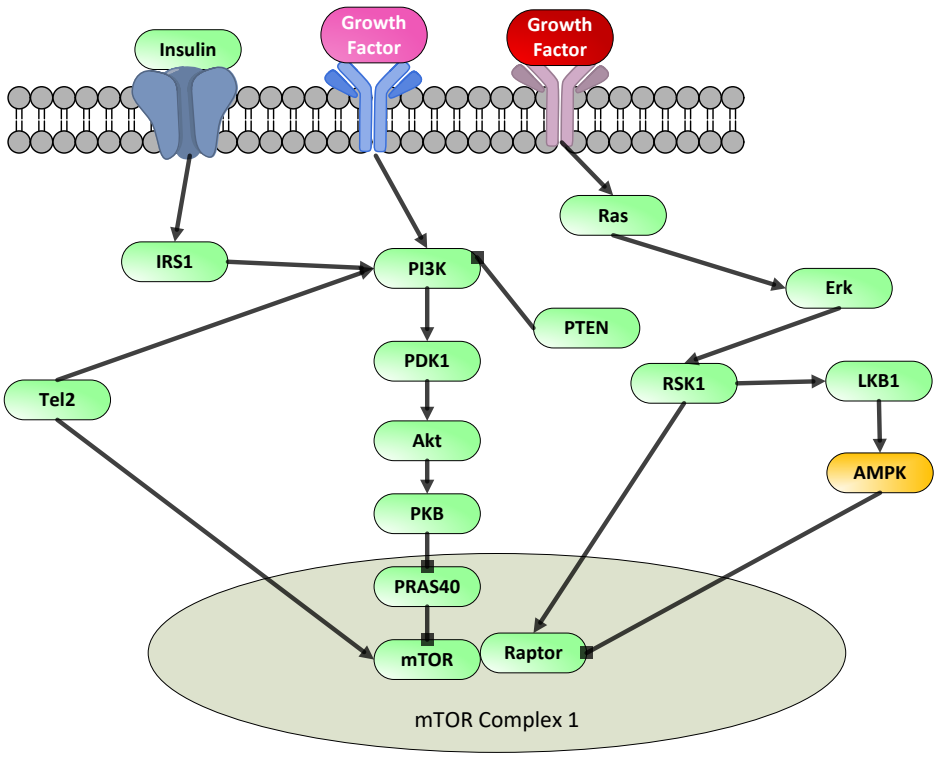
The protein encoded by this gene belongs to a family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation. This protein acts as the target for the cell-cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex.

Now mTOR by itself plays a role only when conjugated with other products, namely those generating mTORC1 and mTORC2. We now briefly explain the structure of each of these two.

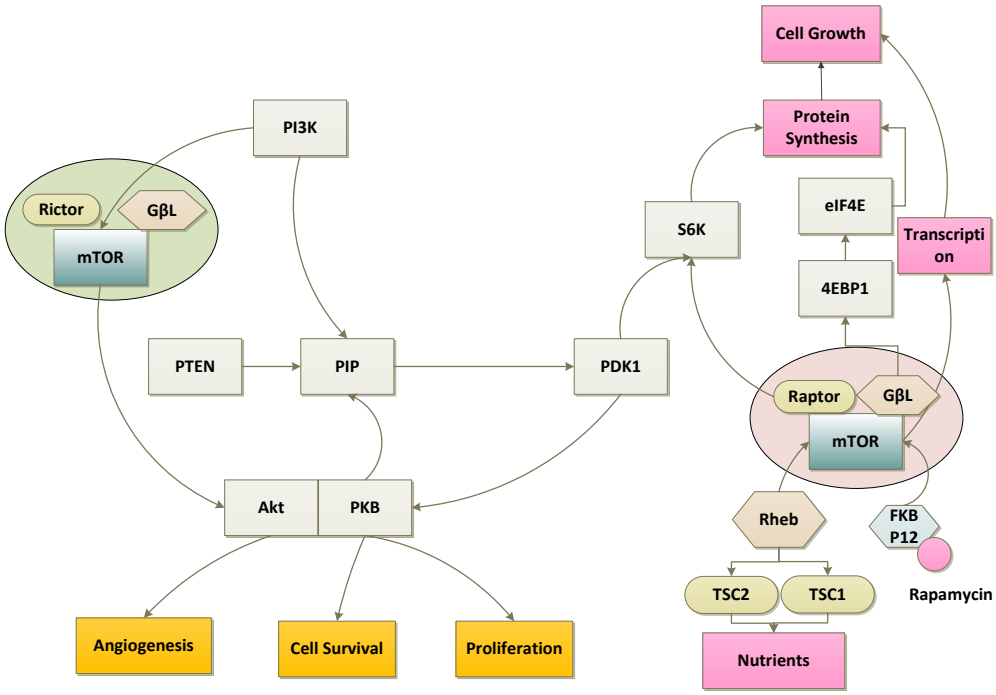
mTOR is a control protein that in involved in metabolic related pathways. mTOR, the mammalian target of rapamycin, is a gene product (1p36.2) is a protein which acts in a critical manner in interconnecting the genetic circuits in mammals, and especially man. It fundamentally controls glucose transport and protein synthesis. The pathway depicted below is a modification of the graphic from Weinberg (p 785) which shows mTOR in its two modes, one with Raptor assisting and one with Rictor. The Rictor/mTOR mode activates the Akt pathway via the placement of a phosphate and this manages the protein synthesis portion. The inclusion of rapamycin will block the Raptor/mTOR path and reduce the protein synthesis and cell growth portion. The inhibitory effect on Akt/PKB by rapamycin is assumed to be the main factor in its anti-cancer effects.

We depict the mTOR C1 pathway below:

[12] https://www.ncbi.nlm.nih.gov/gene/2475



The following chart presents a more complex version of the mTOR C1 pathway (Raptor). This allows us to best understand the complex interactions. The mTOR C1 and C2 pathways are depicted in the combined chart below:



Looking at the complexity of the mTOR pathway it presents an interesting one for addressing PCa. Kinkaide et al (2008) indicate:

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (PTEN) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway. In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP.

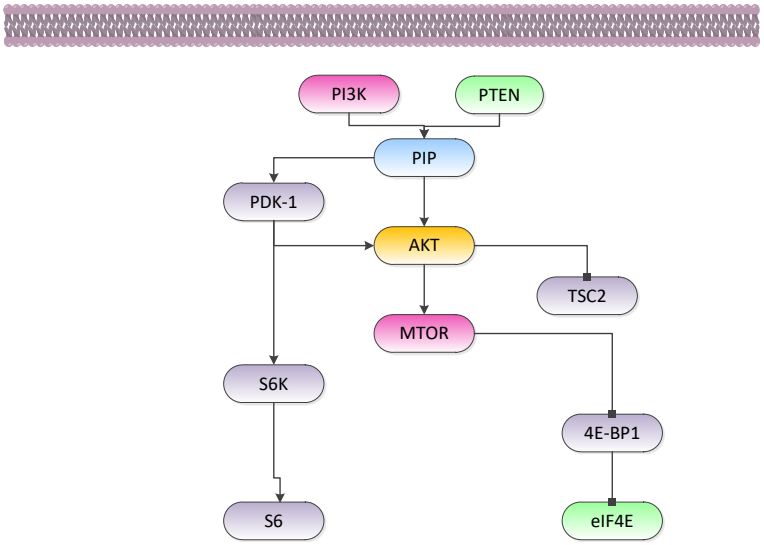
Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/ or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone- refractory disease. These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

Kinkaide et al also demonstrate the creation of HGPIN via their work. This represents another pathway of HGPIN to PCa. LoPiccolo et al state:

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer.

Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We (show) how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

LoPiccolo et al show the more simplified pathway as follows:

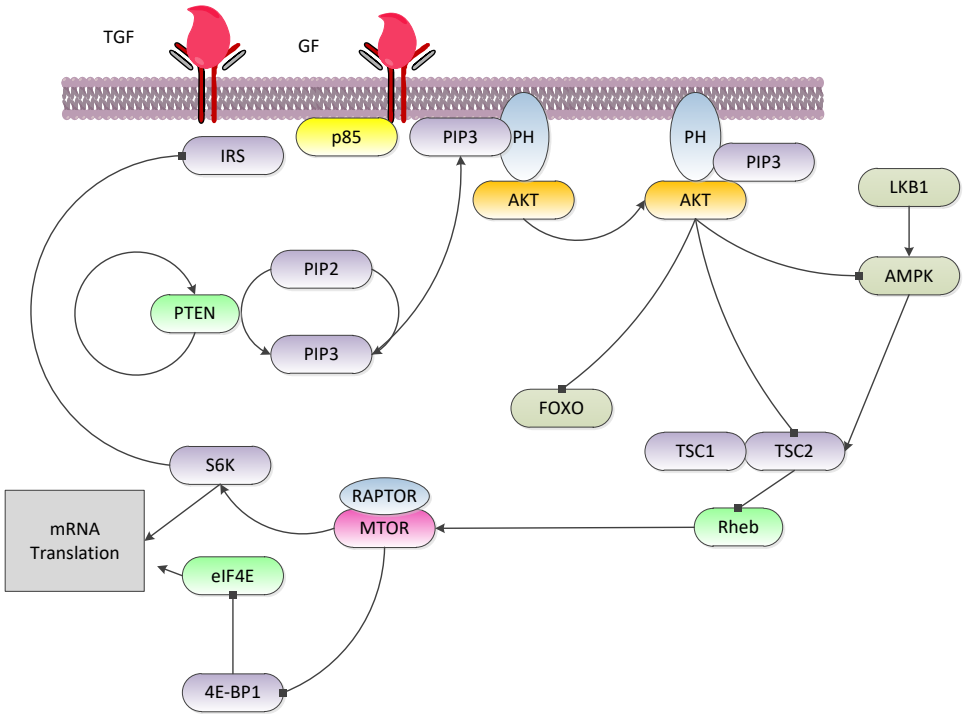


As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. Nelson et al (2007) have demonstrated similar results as well.

Other researchers have also posited other simple models. We demonstrated the one by Hay as has been stated:

The downstream effector of PI3K, Akt, is frequently hyperactivated in human cancers. A critical downstream effector of Akt, which contributes to tumorigenesis, is mTOR. In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, acting as a brake upstream of Akt, and TSC1/TSC2 heterodimer, acting as a brake downstream of Akt and upstream of mTOR.

In the absence of the TSC1/TSC2 brake, mTOR activity is unleashed to inhibit Akt via an inhibitory feedback mechanism. Two recent studies used mouse genetics to assess the roles of PTEN and TSC2 in cancer, underscoring the importance of Akt mTOR interplay for cancer progression and therapy.



The Baldo et al model is quite similar to the Weinberg model shown initially. It clearly demonstrates the overall controlling influence of mTOR. As Baldo et al state:

There is a great body of evidence supporting consideration of the mTOR signaling system as an important network in cell regulation, differentiation and survival. mTOR is a sensor of mitogen, energy and nutritional levels, acting as a "switch" for cell-cycle progression from phase G1 to phase S.

The antibiotic Rapamycin, a potent mTOR inhibitor, has been known to the National Cancer Institute and recognized for its potential anticancer properties since the 1970s. The observation that cell lines from different cancer types exposed to low doses of Rapamycin underwent cell-cycle arrest in phase G1, provided the basis for considering mTOR as a target for cancer therapy.

Development of mTOR inhibitor compounds has proceeded empirically due to the lack of understanding of the precise molecular targets and the required dose of the new compounds . The development of Rapamycin analogs ("Rapalogs"), but also of other, structurally different, mTOR inhibitors, was directed at the selection of specific cancer type sensitivity and an optimization of pharmaceutical forms.

To give an example, Temsirolimus revealed clinical responses in patients with renal cell carcinoma in advanced stage. Temsirolimus was approved by the FDA on May 2007 for this therapeutic use and is being investigated in clinical trials for other cancer types (breast cancer, lymphoma, renal cancer, glioblastoma); significantly there are a considerable number of clinical studies involving mTOR inhibitors currently active worldwide…

The mTOR pathway controls cell size and cellular proliferation*.…nutrient metabolism, mRNA translation and cell survival control. Disruption of TOR leads to early embryonic death in flies and mammalian cells, indicating mTOR plays an important role in regulating cell survival. … deregulation of several mTOR components leads to modified cell proliferation patterns and, on the other, that many mTOR components are deregulated in several human cancers.*

*… **Therefore, inhibition of mTOR leads to slowing or arrest of cells in the G1 phase**. Translational control may have an important role in the balance of cell survival and death, and hence for apoptosis. Importantly, components of mTOR are deregulated in some human cancers, for example, breast and colon. Alteration of PI3-K/Akt is frequently observed in head and neck cancer .*

PTEN, a phosphatase that acts on PIP3 to convert it to PIP2, normally regulates the mTOR pathway negatively, and shows decreased activity in some tumors. A strong relation seems to exist between the sensitivity to the effect of Rapamycin and PTEN loss or deregulation. PTEN is frequently mutated in several cancers and in cancer-like syndromes like Cowden and Proteus syndromes…

Loss of PTEN function can occur in 26-80% of endometrial carcinomas*, …recent studies of human prostate cancer have shown that loss of PTEN is strongly associated with more aggressive cancers. The relationship between PTEN status and sensitivity to rapalogs has been questioned by several investigators. Some attention has recently been dedicated to the role of the mTORC2 complex in the mTOR pathway.*

In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, is predicted to integrate signals derived from nutrients, cellular energy status and hypoxia into a common growth regulatory signal to the mTORC1 complex.

As Easton and Houghton state:

Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed rapamycin analogs (rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types in vitro, and tumor models in vivo.

Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.

The other observation here is that we often find multiple characterizations of the pathways. Namely there is no canonical form, and often a pathway is depicted to demonstrate a specific protein function. Thus we may see an emphasis on one set of proteins while others are neglected. As much as we currently attempt to unify this process we are left somewhat adrift in model development at this stage. This can be exemplified by now looking at the next section on LKB1. There we show its control over PTEN whereas in an earlier model we have it controlling AMPK. In reality there are multiple links as we have discussed. The literature can be even more confusing on this issue as well.

As Mendelsohn et al state:

It is now widely accepted that mTORC1 positively controls an array of cellular processes critical for growth, including protein synthesis, ribosome biogenesis, and metabolism, and negatively influences catabolic processes such as autophagy—all of which have roles in cancer pathogenesis. Elucidating the key downstream targets of mTORC1 driving these events is an intense area of research.

Originally, much of the study of mTOR relied on experiments in which rapamycin was used acutely to inhibit mTOR (which we now know was mTORC1) in cultured cells. This led to extensive characterization of the best known mTORC1 substrates eiF-4E-binding protein 1(4E-BP1) and S6 kinase 1 (S6K1), both of which regulate protein synthesis.3 In the unphosphorylated state, 4E-BP1 binds and inhibits the cap-binding protein and translational regulator eIF4E. When phosphorylated by mTOR, 4E-BP1 is relieved of its inhibitory duty, promoting eIF4E interaction with the eIF4F complex and the translation of capped nuclear transcribed mRNA.

Following co-regulatory phosphorylation by mTORC1 and another kinase called phosphatidylinositol 3-dependent kinase 1 (PDK1), S6K1 positively affects mRNA synthesis at multiple steps including initiation and elongation by phosphorylating several translational regulators. Although the preponderance of evidence indicates that S6K1 and 4E-BP1 are directly phosphorylated by mTOR, an unidentified phosphatase activity may also be involved in their regulation. For example, the rapamycin-sensitive phosphorylation site on S6K1 is rapidly dephosphorylated (i.e., within minutes) of exposure to the drug.

They continue:

Conditions that inhibit growth, such as decreased energy, low oxygen, and insufficient nutrients, are associated with the harsh microenvironment of poorly vascularized tumor. The ability of cancer cells to overcome these adverse conditions would promote tumor growth, putting the desensitization of mTORC1 signaling in the spotlight as a potential mechanism cancer cells could exploit to enhance their viability. Whether mutations in the amino acid– and glucose-sensing pathway that activates mTORC1 exist in cancer is not known. Mutations in the growth factor inputs to mTORC1 are prominent in cancer.

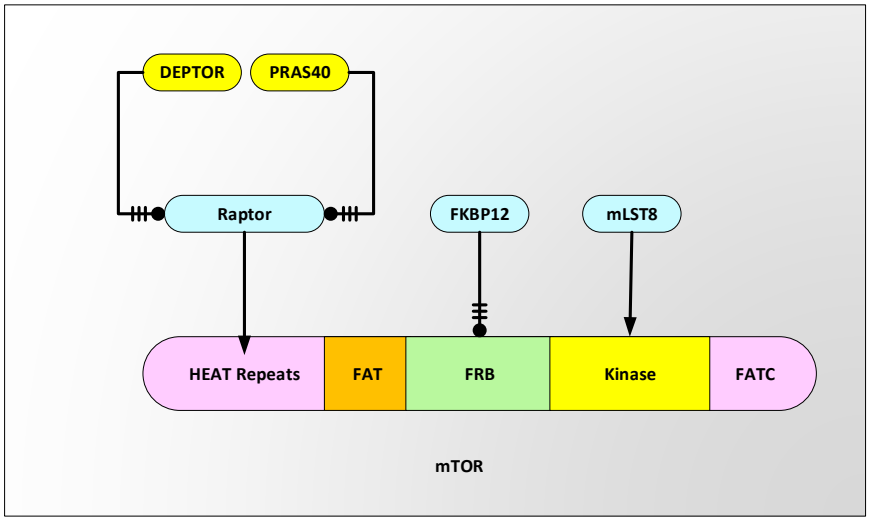
For example, mutations causing loss of PTEN function or oncogenic activation of PI3K or AKT are associated with many aggressive human cancers (Table 12-1).17-20 The findings that AKT

promotes mTORC1 activity through TSC and PRAS40 suggest that cancers with elevated PI3K-AKT signaling may in part thrive because of an mTORC1-driven growth advantage. Activation of PI3K-AKT signaling also facilitates nutrient uptake by cells, which indirectly contributes to mTORC1 activity by localizing mTORC1 to lysosomes.

Therefore, understanding the contribution and relevance of mTORC1 signaling in the progression of cancers with aberrant PI3K-AKT signaling is an important area of research.

2.3.1 mTORC1

As we noted earlier mTORC1 has the most significant set of impacts on cell stability. Also as we noted there are upstream and downstream influences generated by this complex. We start with the structure of the mTORC1 complex as noted below:



The mTOR protein is composed of five sections, including the kinase element. The HEAT Repeats, as noted by Neuwald and Hirano are:

HEAT repeats correspond to tandemly arranged curlicue-like structures that appear to serve as flexible scaffolding on which other components can assemble. Using sensitive sequence analysis techniques we detected HEAT repeats in various chromosome-associated proteins, including four families of proteins associated with condensins and cohesins, which are nuclear complexes that contain structural maintenance of chromosome (SMC) proteins.

RAPTOR is the regulatory associated protein of mTOR[13]. RAPTOR is an mTOR binding protein.

As Saxton and Sabatini have noted:

[13] https://www.ncbi.nlm.nih.gov/gene/57521

In order to grow and divide, cells must increase production of proteins, lipids, and nucleotides while also suppressing catabolic pathways such as autophagy. mTORC1 plays a central role in regulating all of these processes and therefore controls the balance between anabolism and catabolism in response to environmental conditions... the critical substrates and cellular processes downstream of mTORC1 and how they contribute to cell growth.

Most of the functions discussed here were identified and characterized in the context of mammalian cell lines, while the physiological context in which these processes are important will be discussed in greater detail below.

Protein Synthesis mTORC1 promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). mTORC1 directly phosphorylates S6K1 on its hydrophobic motif site, Thr389, enabling its subsequent phosphorylation and activation by PDK1.

S6K1 phosphorylates and activates several substrates that promote mRNA translation initiation, including eIF4B, a positive regulator of the 50cap binding eIF4F complex. S6K1 also phosphorylates and promotes the degradation of PDCD4, an inhibitor of eIF4B, and enhances the translation efficiency of spliced mRNAs via its interaction with SKAR, a component of exon-junction complexes.

The mTORC1 substrate 4EBP is unrelated to S6K1 and inhibits translation by binding and sequestering eIF4E to prevent assembly of the eIF4F complex. mTORC1 phosphorylates 4EBP at multiple sites to trigger its dissociation from eIF4E, allowing 50cap-dependent mRNA translation to occur.

Although it has long been appreciated that mTORC1 signaling regulates mRNA translation, whether and how it affects specific classes of mRNA transcripts has been debated. Global ribosome footprinting analyses, however, revealed that, while acute mTOR inhibition moderately suppresses general mRNA translation, it most profoundly affects mRNAs containing pyrimidine-rich 50 TOP or ''TOP-like'' motifs, which includes most genes involved in protein synthesis

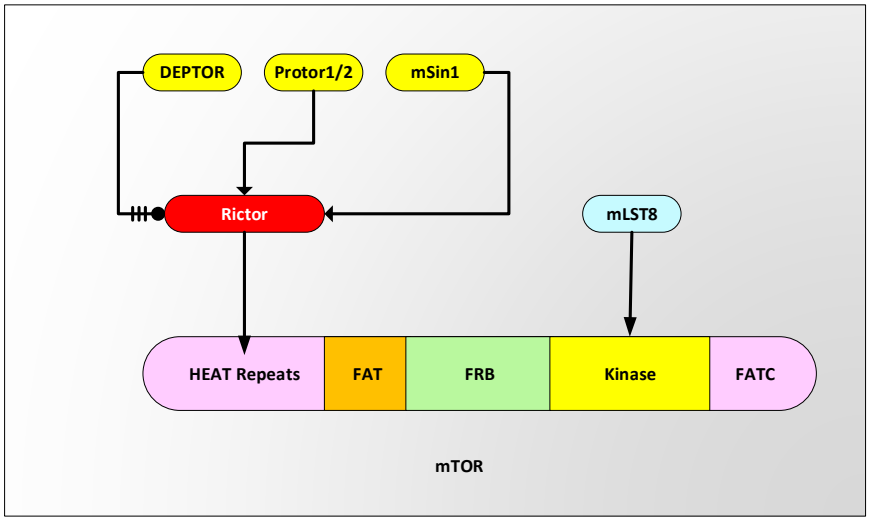
Now the upstream influencers or drivers are detailed below from Seeboeck et al:

mTORC1 Upstream	
Rapamycin	rapamycin
FKBP12	FK506-binding protein 12 kDa
TSC	tuberous sclerosis complex
Rheb	Ras homolog enriched in brain
IGF-1 pathway	insulin/insulin like growth factor
AKT	AKT serine/threonine kinase
mTORC2	promotes dissociation of PRAS40 from mTORC1.
Wnt	Wnt
TNFα 1	tumor necrosis factor α
AMPK	5'-AMP-activated protein kinase
REDD1	regulated in development and DNA damage responses 1

The above each in their own manner effects the actions of mTORC1. Rapamycin is a major driver when present. Some of these are exogenous to the cell itself such as the growth factors and others are part of the cell normal pathway. Note that mTORC2 has a driving factor as well. We shall briefly explore that next.

2.3.2 mTORC2

Now we consider mTORC2. From Seeboeck et al the structure appears as below:



Rictor is akin to Raptor. We see the same underlying mTOR base elements and then the complex binding to create the multiprotein complex. Now the drivers or upstream elements are shown below. Like mTORC1, it also is a driver here.

mTORC2 Upstream	
Rapamycin	rapamycin
FKBP12	FK506-binding protein 12 kDa
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
AKT	AKT serine/threonine kinase
mTORC1	Negative feedback loop between mTORC1 and insulin/PI3K signaling

Saxton and Sabatini have noted the downstream effects of mTORC2:

While mTORC1 regulates cell growth and metabolism, mTORC2 instead controls proliferation and survival primarily by phosphorylating several members of the AGC (PKA/PKG/PKC) family of protein kinases.

The first mTORC2 substrate to be identified was PKCa, a regulator of the actin cytoskeleton. More recently, mTORC2 has also been shown to phosphorylate several other members of the

PKC family, including PKCd, PKCz, as well as PKCg and PKCε, all of which regulate various aspects of cytoskeletal remodeling and cell migration.

The most important role of mTORC2, however, is likely the phosphorylation and activation of Akt, a key effector of insulin/ PI3K signaling.

Once active, Akt promotes cell survival, proliferation, and growth through the phosphorylation and inhibition of several key substrates, including the FoxO1/3a transcription factors, the metabolic regulator GSK3b, and the mTORC1 inhibitor TSC2.

However, while mTORC2- dependent phosphorylation is required for Akt to phosphorylate some substrates in vivo, such as FoxO1/3a, it is dispensable for the phosphorylation of others, including TSC2. Finally, mTORC2 also phosphorylates and activates SGK1, another AGC-kinase that regulates ion transport as well as cell survival.

The mTORC1-dependent shift toward increased anabolism should only occur in the presence of pro-growth endocrine signals as well as sufficient energy and chemical building blocks for macromolecular synthesis. In mammals, these inputs are largely dependent on diet, such that mTORC1 is activated following feeding to promote growth and energy storage in tissues such as the liver and muscle but inhibited during fasting conserve limited resources. Here, we discuss the cellular pathways upstream of mTORC1 and the mechanisms through which they control mTORC1 activation.

2.4 REDD1

From Chang et al:

REDD1 (regulated in development and DNA damage response 1, also known as RTP801/Dig1/DDIT4) was first identified in 2002. It is a stress related protein induced by hypoxia and multiple DNA damage stimuli and is expressed broadly in many human tissues [1]. The gene is located at human chromosome 10q24.33 and is homologous to two Drosophila melanogaster genes of unknown function, Scylla and Charybde, which are designated as Hox targets in the National Institutes of Health genetic sequence database GenBank. As a potent repressor of the mechanistic target of rapamycin in complex 1 (mTORC1), REDD1 regulates cell growth, tumorigenesis, cell aging, and autophagy

2.5 OBSERVATIONS

Talty and Olino in examining the impact of the innate immune system also reflect on metformin. They note:

Several ongoing clinical trials focus on combining metabolism-targeted agents with immunotherapy treatments. Many of these trials exploit the use of previously approved drugs such as metformin and rosiglitazone which are used in the treatment of diabetes and alter

downstream metabolic pathways. For example, metformin decreases peripheral insulin resistance by inhibiting mitochondrial respiration and activating AMPK.

Activated AMPK inhibits metabolic processes such as gluconeogenesis and lipogenesis and stimulates glucose uptake and fatty acid oxidation thus affecting additional pathways tied in with immunometabolism. Metformin can also target mTOR, insulin-like growth factor, and mitogen-activated protein kinase (MAPK) pathways. In various preclinical studies, metformin has been shown to potentiate antitumor immunity more directly by promoting STING and Hippo signaling, PD-L1 degradation, and a reduction in tumor hypoxia. Rosiglitazone activates PPARγ and has had similar preclinical results.

3 PROSTATE CANCER

We briefly examine prostate cancer[14]. This is a frequent cancer among men and it can generally be treated surgically or at time by radiation. The prostate can be monitored by measuring the PSA value but it has been deemed of less than perfect value. Additional measurements such as % Free, velocity, and PSA per unit volume can be used. Many other tests have become available over the years. Following a suspicious test one then goes through an mpMRI with a resulting PIRAD score. We have argued that the PIRAD may be also of limited value if multiple prior biopsies have been performed since the resulting residual scar tissue can appear as a lesion in diffusion weighted scans. The next step would be a biopsy targeting areas found suspicious on the mpMRI. However even then the assessment can be less than what is actually obtained upon excisional biopsy. Finally, it is often the case that the lesion can be genomically analyzed. The current problem here is that large number of cells are processes and that artifact may suppress the identification of key genes. The use of single cell sampling the Next Gen sequencing can be exceptionally useful but may be costly.

3.1 HISTOLOGY

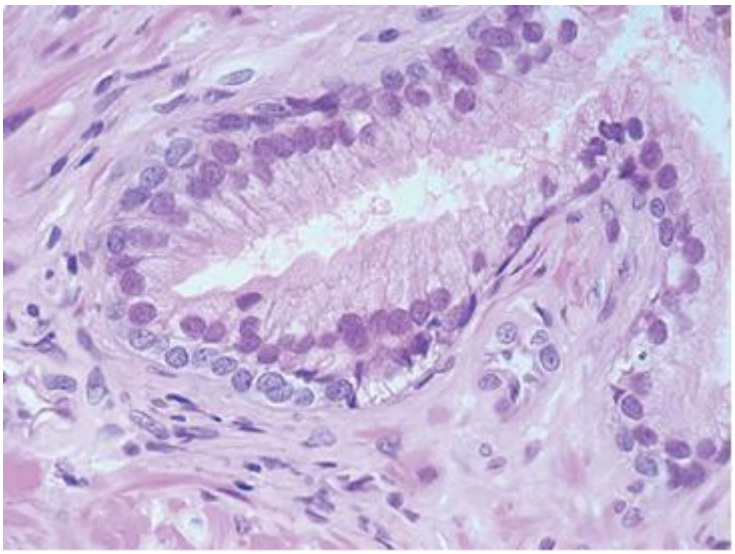
We provide a brief overview of the histological finding using the Gleason approach. We rely upon the excellent reference by Epstein from which several of the images have been used.

3.1.1 Normal Histology

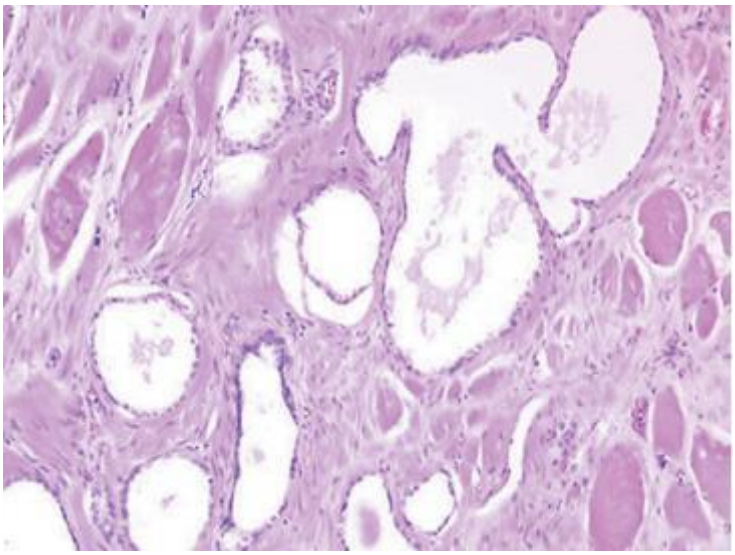
The prostate cellular structure is depicted below. There are approximately 35-50 glands in the prostate, mostly in the peripheral zone and the glands have a lumen in which the prostatic secretions flow, and the glands have basal cells and luminal cells as shown below. The basal cells are dark and the luminal cells are somewhat lighter.

Between the cells is the stroma which includes the blood flow from veins and arteries, the muscle and other stroma elements. Simply stated, the prostate is a collection of the basal/luminal glands scattered about veins, arteries, muscles and nerves.

[14] See https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach This is a Draft book written in 2010 and edited slightly. It is a reasonable view of PCa at that time. Like so much in cancer research details change rapidly.



The figure below depicts a second view of the prostate glands. Again this is with HE stain and under low magnification. The basal cells are clearly see with their dark stains and the luminal stand above them. The stroma is fairly well articulated in this slide.



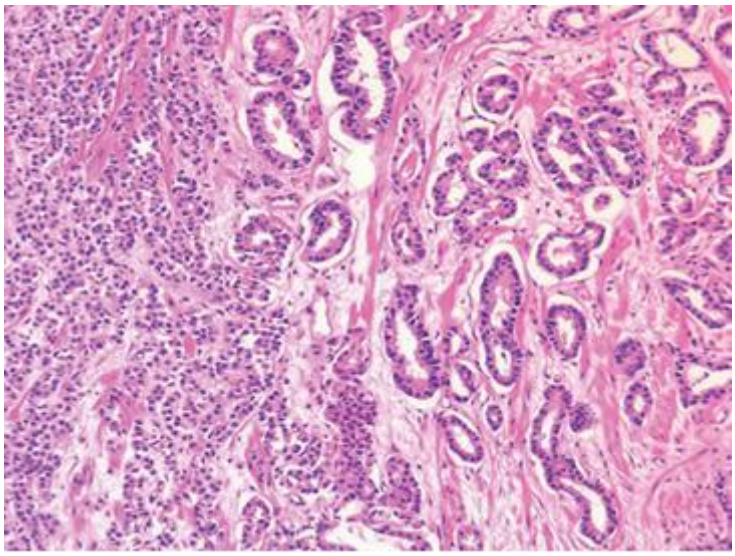
The normal prostate then is merely a collection of glands, glands composed of basal and luminal cells, with open glandular portions, the white areas above. As we noted before these glands emit various proteins and are an integral part of the male reproductive system.

3.1.2 PCa Grading

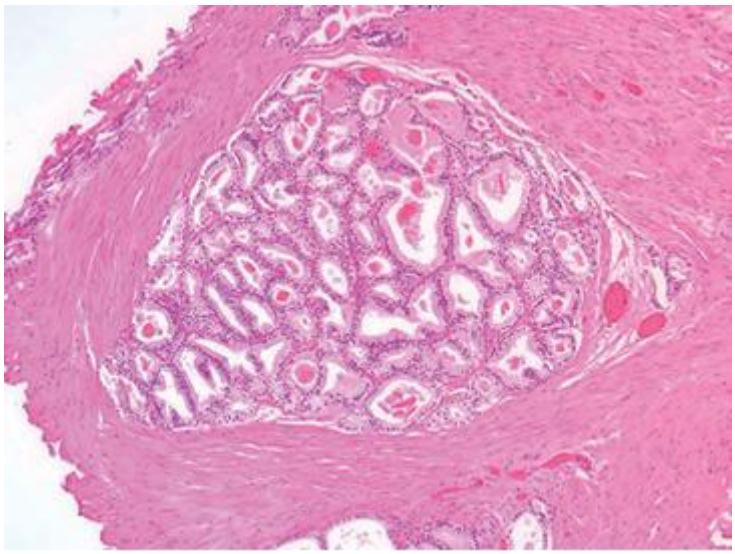
We present the grading system developed by Gleason. On the one hand this has been used as a gold standard for ascertaining future progress and yet it is still just a morphological tool. It fails to determine the pathways and regulators in a cell by cell basis.

3.1.2.1 Gleason 1

The following is a Gleason 1 grade tumor. Note that there are a proliferation of small glandular like clusters with dark basophillic stains and they are separate and have clear luminal areas. Gleason 1 is generally composed of many single and separate and closely packed glands of well circumscribed uniforms glands. One rarely sees Gleason 1 grade tumors, and they are often found as incidental findings when examining for other issues.



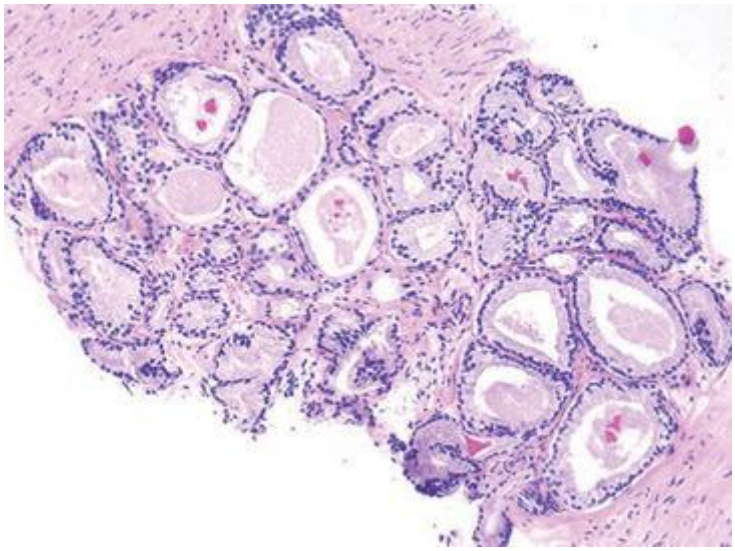
We show another view of a Gleason 1 below. This is especially descriptive of such a form because it appears almost as a single and isolated structure. The interesting question will be if this is PCa then if PCa is clonal is this cluster an aberrant outgrowth of a normal cells, if so which one, and if so is this just one cell growing. It appears that at this stage the intercellular signaling is still trying to function. However the clarity of cell form is being degraded.



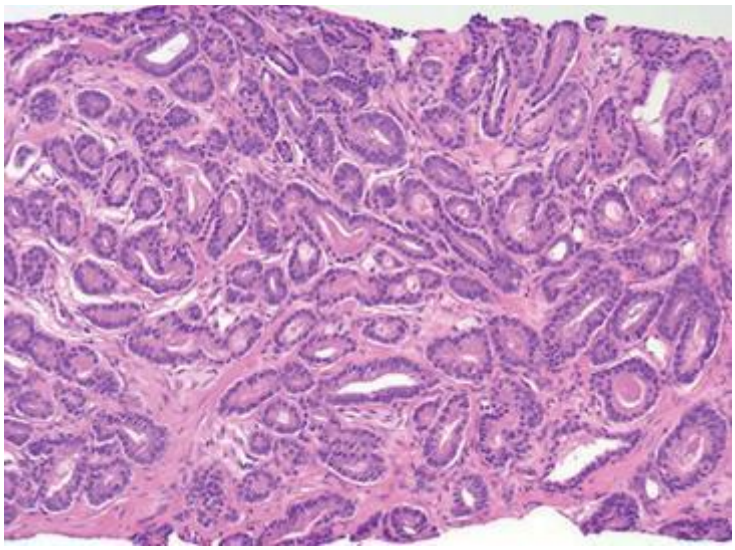
1.1.1.1 Gleason 2 and 3

Gleason 2 shows many more new glandular like cells but now of varying larger sizes. As Epstein notes: "*Grade 2 … is still fairly circumscribed, at the edge of the tumor nodule there can be minimal extension by neoplastic glands into the surrounding non-neoplastic prostate. The glands are more loosely arranged and not as uniform as Gleason 1*." We see those in the figure below which combines Gleason 2 and 3.

Gleason 3 is often composed of single glands. The Gleason 3 infiltrates in and amongst the non-neoplastic glands. Gleason 3 still can be seen as a separate gland and there are no single cells starting to proliferate. In Gleason 3 we still have some semblance of intercellular communications and coordination, albeit with uncontrolled intracellular growth. Again in the figure below we see both the smaller 2 and the larger 3 with gland structure being preserved and no separate cells proliferating.

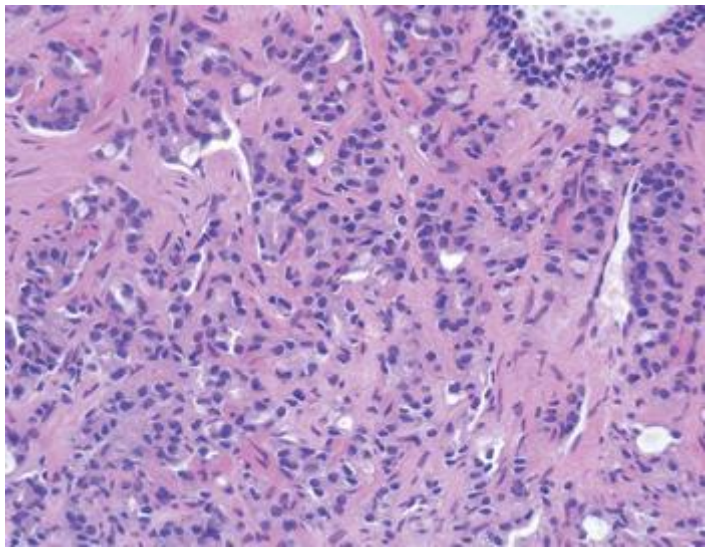


A Gleason 3 throughout is shown below.

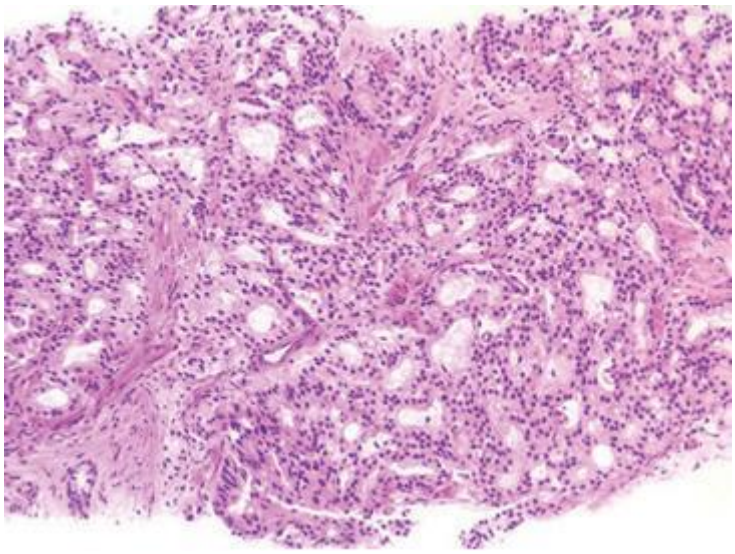


1.1.1.2 Gleason 4

Gleason 4 consists mostly of cribiform cells (perforated like a sieve) or fused and ill-defined glands with poorly formed glandular lumina. The glands appear to start to "stick" together. A Gleason 4 with a Gleason 3 is shown below. Note the sieve like structure and the closing of the glands.

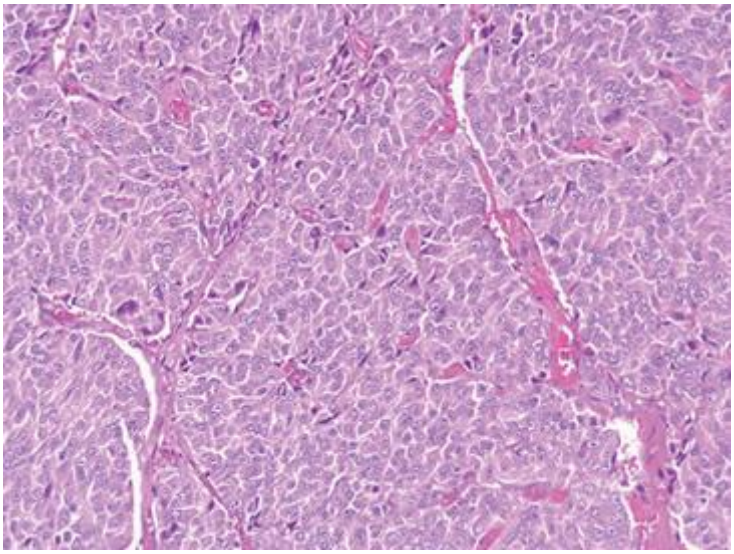


A Gleason all 4 is shown below. Note that the cells are sticking closed and the entire mass appears as a sieve-like mass.



1.1.1.3 Gleason 5

Gleason 5 is a complete conversion to independent malignant cells. They have lost all intercellular coordination. As shown below it is a mass or mat or sheet of independent cancer cells and it has lost any of the sieve like structures. There may also appear to be some necrosis



3.1.3 Gleason Summary

The Gleason scores are then determined by taking the predominant type and adding it to the secondary type. Thus a 4+3 yields a Gleason combined 7 but it is 4+3 and that is more aggressive than say a 3+4 with the same total score.

We repeat the grading commentary below.

Gleason 1	Gleason 2	Gleason 3	Gleason 4	Gleason 5
Many acini with no basal layers and large nucleoli. Closely packed clumps of acini.	Many very small single separate glands (acini) with no basal layer and large nucleoli . Glands, acini, are more loosely arranged and not close packed.	Many small microglands extending throughout the stroma and out of the normal gland structure	Glands are now spread out and fused to one another throughout the stroma.	No gland structure seen, all luminal cells throughout the stroma with large nucleoli.

3.2 PROGRESSION

We now examine the classic paradigm for PCa progression. The principal player is the androgen receptor, AR. As Kokal et al have recently noted:

The androgen receptor (AR) plays a leading role in the control of prostate cancer (PCa) growth.

Interestingly, structurally different AR antagonists with distinct mechanisms of antagonism induce cell senescence, a mechanism that inhibits cell cycle progression, and thus seems to be a key cellular response for the treatment of PCa. Surprisingly, while physiological levels of androgens promote growth, supraphysiological androgen levels (SAL) inhibit PCa growth in an AR-dependent manner by inducing cell senescence in cancer cells.

Thus, oppositional acting ligands, AR antagonists, and agonists are able to induce cellular senescence in PCa cells, as shown in cell culture model as well as ex vivo in patient tumor samples.

This suggests a dual AR-signaling dependent on androgen levels that leads to the paradox of the rational to keep the AR constantly inactivated in order to treat PCa. These observations however opened the option to treat PCa patients with AR antagonists and/or with androgens at supraphysiological levels. The latter is currently used in clinical trials in so-called bipolar androgen therapy (BAT).

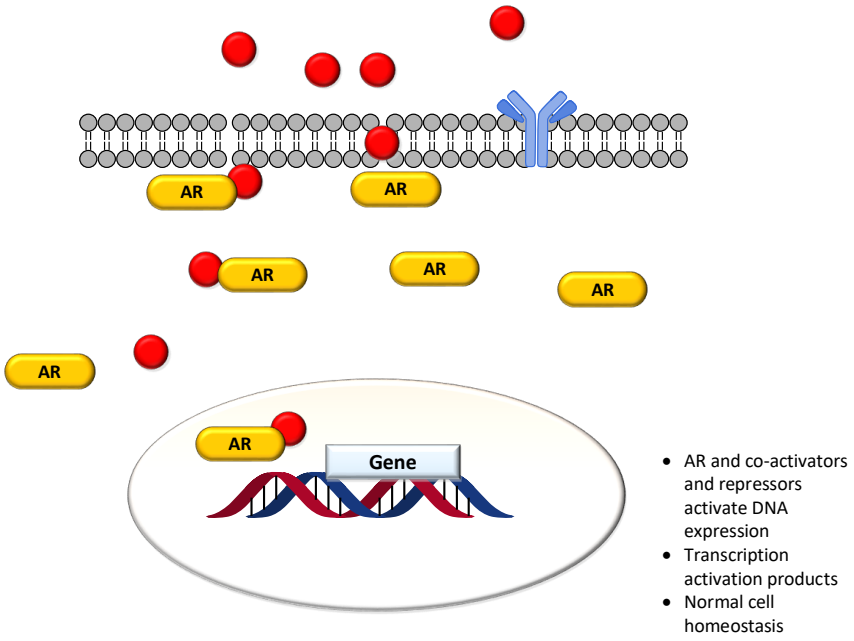
Notably, cellular senescence is induced by AR antagonists or agonist in both androgen-dependent and castration-resistant PCa (CRPC). Pathway analysis suggests a crosstalk between AR and the non-receptor tyrosine kinase SrcAkt/PKB and the PI3K-mTOR-autophagy signaling in mediating AR-induced cellular senescence in PCa. In this review, we summarize the current

knowledge of therapeutic induction and intracellular pathways of AR-mediated cellular senescence. …

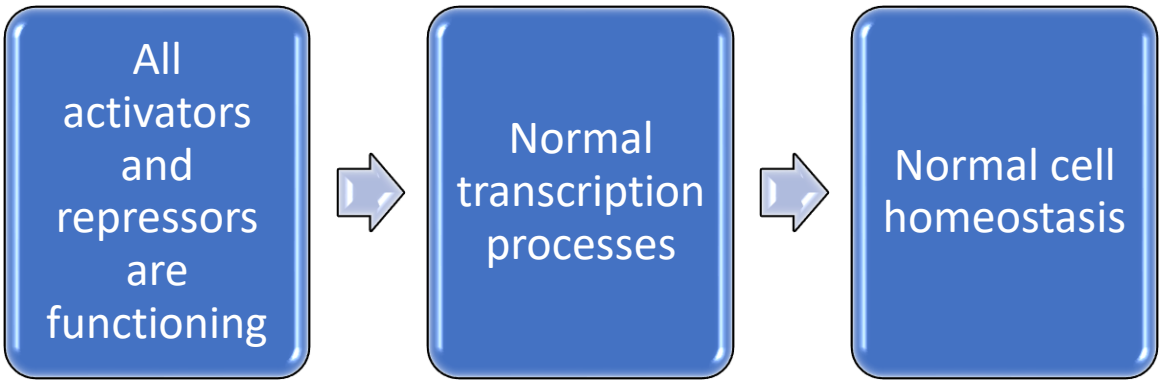
In conclusion, both androgens at supraphysiological levels and AR antagonists induce cellular senescence in PCa.

This important AR pathway is mediated by membrane and cytosolic transduction factors including PI3K, Src family, Akt and mTOR. AR was shown previously to interact in a non-genomic and rapid signaling with Src and Akt. Analyzing AR ligand-induced cell senescence, the activation of these factors was however also observed after many days of AR ligand treatment. Therefore, it is suggested that the AR interacts with these factors at the non-genomic level, also in a long-term manner, which eventually changes the transcriptome landscape. Interestingly, despite both ligands inducing cancer cell senescence, AR agonist and antagonist seem to induce a distinct pro-survival pathway. Therefore, targeting senescent PCa cells, the specific pro-survival pathway should be known in order to use a particular senolytic compound

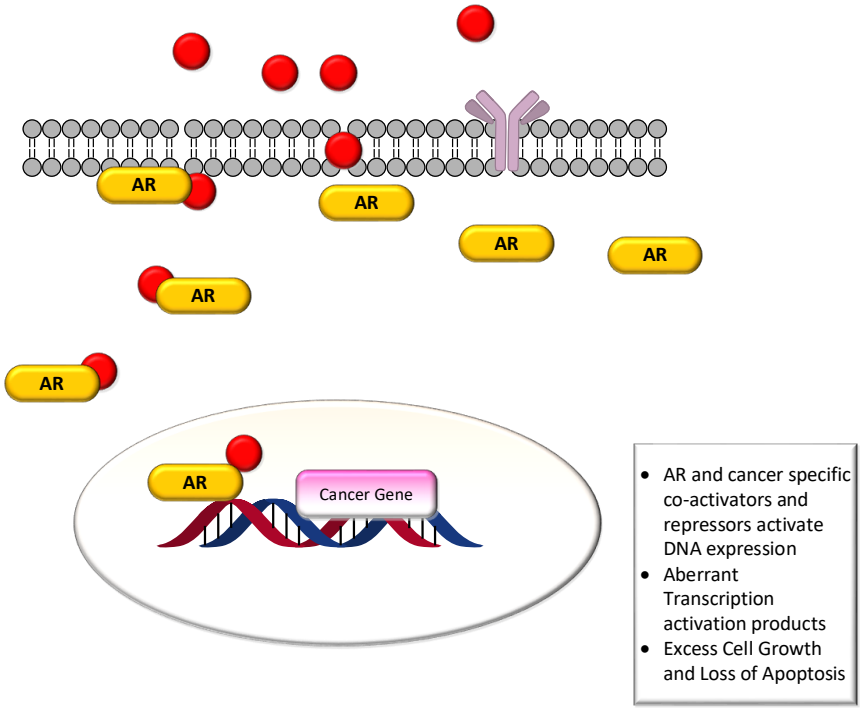
In normal AR operations, we show below the Testosterone coming into the cell and then it binds with the AR. It is this normal bonding which gives the AR the ability to manage a significant portion of the normal growth of the prostate cell. We use the simplified graphics a below to demonstrate. The Normal Process thus is as follows and it is one of classic homeostasis. Androgens arrive in the cell, the AR collects them, and the net effect is a homeostatic gene activity.



The normal benign process then proceeds as follows:



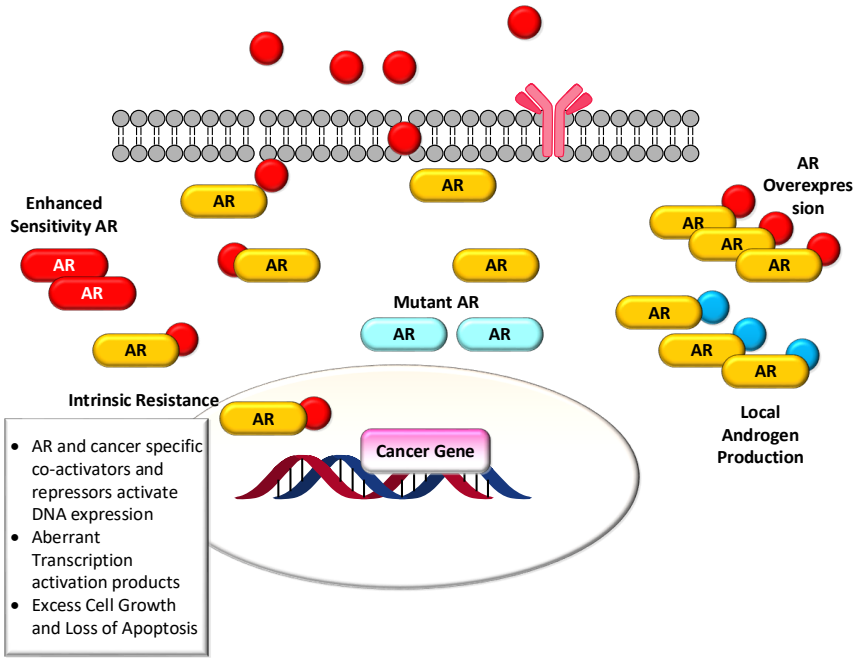
In the case of cells having exhibited PCa we see the AR playing the role of excess growth enhancer. Namely the process is as before but the AR becomes over activated and thus cell proliferation occurs. The issue here is twofold. First a set of gene changes occur (mutations, methylations, etc) that starts aberrant cell proliferation. Second the cells use the androgens to assist in this process using the AR as a mechanism to transport.



As is best understood, the progression towards AR resistant PCa follows the path shown below.

Enhanced AR Sensitivity	•The reduced androgens interact with increased ARs so that the efficiency is increased
AR Mutations	•The ARs are mutated so that they are more efficient
Alternative Signalling Cascades	•The signalling channels on the cells surface may be modified
AR Overexpression	•The ARs may have an ability to excessively produce excess receptors
Local Androgen Expression	•Local internal cytoplasmic androgen production may occur
Intrinsic Resistance	•Cancer cells may be those with low intrinsic resistance

When the cell becomes refractory to AR functions, there may at first be AR overexpression and then a set of PCa specific receptors develop which result in metastatic grown as depicted below. Here new versions of an AR are generated that can do their functions without the need of androgens. The cells can become self-sustaining and proliferate without limitation. In effect mutant ARs are generated that allow for continue proliferation devoid of any androgens.



The process then flows as follows:

These three stages, benign, androgen responsive malignant, and androgen refractory malignant give the general progression of PCa. We can see this general process in all PCa. The objective is first to remove all the tumor, if not then manage via androgen suppression, and if that is no longer possible seek a multiple set of therapeutic options.

3.3 GENOMICS

There are a multiplicity of genes associated with PCa. Wang et al have recently focused on a few:

*Expression of **TNF-α** and **NF-κB-P65** was upregulated, while E-cadherin expression was downregulated in PCa specimens with a high Gleason score …*

*TNF-α expression and NF-κB-P65 expression were mainly localized in the cytoplasm and nuclei of luminal cells of prostate carcinoma glands, while **E-cadherin** protein was localized in the cell membrane and cytoplasm of luminal cells of normal prostate glands and of prostate carcinoma cells. The expression of TNF-α and NF- κB-P65 was significantly increased in PCa cases, showing a highest expression in PCa cases with the high Gleason score.*

However, E-cadherin expression was significantly decreased in PCa cases, showing a lowest expression with the high Gleason score.

We see the E-cadherin issue arise in many cancers. It leads to the EMT issue, namely the malignant cells lose their footing and start wandering where they do not belong.

Metformin inhibited TNF-α-induced epithelial– mesenchymal transition in PC3 cells.

This is a critical observation. If one can stop the EMT process then perhaps the cells can be delimited to the organ and not metastasize.

First, the effects of metformin on TNF-α-induced EMT in PC3 prostate cancer cells were examined. After being treated with TNF-α for 72 h, we found that the morphology of PC3 cells exhibited significant changes, and these PC3 cells exhibited more features of mesenchymal fibroblast-like and fusiform.

Exposure of PC3 cells to TNF-α significantly reduced the expression of the epithelial marker E-cadherin ($P<0.05$) and induced the expression of the mesenchymal markers N-cadherin and Vimentin ($P<0.05$), which are changes characteristic of EMT. However, exposure of PC3

prostate cancer cells to TNF-α combined with metformin significantly restored the typical epithelial cobblestone morphology, restored the expression of the epithelial marker E-cadherin ($P<0.05$), and significantly downregulated the mesenchymal markers N-cadherin and Vimentin ($P<0.05$). Furthermore, as shown by immunofluorescence, E-cadherin was mainly expressed in the cell membrane, and its expression was downregulated by TNF-α but upregulated after combined treatment with metformin

Metformin suppressed TNF-α-induced PC3 cell migration and invasion capacity We examined the effect of TNF-α and metformin on prostate cancer cell migration using a wound healing assay and Boyden chamber invasion assay. *…*

TNF-α promoted the migration and invasion ability of PC3 cells, but metformin significantly attenuated the migration and invasion ability of PC3 cells induced by TNF-α ($P<0.05$), consistent with our observation of the EMT pattern.

Metformin inactivated the NF-κB signaling pathway in PC3 cells …exposure of PC3 cells to TNF-α upregulated the expression of p-IKK, p-IκBα, and NF- κB-P65. However, exposure of PC3 cells to TNF-α and metformin significantly downregulated the expression of p-IKK, p-IκBα, and NF-κB-P65 ($P<0.05$). Furthermore, as shown by immunofluorescence, NF-κB-P65 was mainly expressed in the cytoplasm and nuclei of cells.

Metformin downregulated the expression of NF-κB-P65 and inhibited its translocation into the nucleus. Moreover, the effects of BAY11-7082 on PC3 prostate cancer cells were examined. Consistent with the metformin results, BAY11-7082 significantly downregulated the expression of p-IKK, p-IκBα, and NF-κB-P65…..

TNF-α induces a series of inflammatory responses that further activate the NF-kB signaling pathway. Interestingly, this proinflammatory feedback loop also participates in prostate cancer. TNF-α significantly upregulated the expression of p-IKK, p-IκBα, and NF-κB-P65. However, metformin significantly lowered the TNF-α- induced expression of p-IKK, p-IκBα, and NF-κB-P65. Furthermore, metformin also inhibited NF-κB-P65 translocation into the nucleus. This study found that TNF-α promoted the EMT process, which was inhibited by BAY11-7082, an inhibitor of NF-κB.

Consistent with our immunohistochemistry results in human prostate cancer tissues, TNF-α and NF-κB-P65 were positively correlated with the Gleason score, while E-cadherin expression was negatively correlated with the Gleason score. Therefore, we speculated that activation of the NF-κB signaling pathway might be involved in the EMT process in prostate cancer and prostate cancer progression and metastasis. Metformin might suppress the EMT process in prostate cancer and inactivate the TNF-α-induced NF-κB signaling pathway.

In conclusion, upregulation of TNF-α expression, activation of the NF-κB signaling pathway, and induction of the EMT process were involved in prostate cancer progression. In addition, metformin could suppress TNF-α- induced EMT in prostate cancer, potentially by inactivating the NF-κB signaling pathway.

Epstein comments regarding germ-line testing. He begins with germ line gene expressions:

Germ line mutations in BRCA2 in the past were not considered a significant factor in prostate cancer as only 1% to 3% of localized prostate cancer have BRCA2 germ line mutations.39–42 However, germ line mutations in DNA damage repair genes, most commonly BRCA2 alterations, are present in 8% to 16% of metastatic prostate cancer patients.

Inherited BRCA2 mutations have been described in 3% to 5% of patients with advanced prostate cancer. In a study of 3,607 men with a history of prostate cancer that underwent germ line genetic testing that was unselected for family history, stage of disease, or age and was ordered at the discretion of the referring physician, the top 10 genes with positive variants as a percentage of men tested were as follows:

1. ***BRCA2, 4.74%;***
2. ***CHEK2, 2.88%;***
3. ***ATM, 2.03%;***
4. ***MUTYH, 2.37%;***
5. ***APC, 1.28%;***
6. ***BRCA1, 1.25%;***
7. ***HOXB13, 1.12%;***
8. ***MSH2, 0.69%;***
9. ***TP53, 0.66%; and***
10. ***PALB2, 0.56%.***

Positive variants in mismatch repair (MMR) genes (PMS2, MLH1, MSH2, MSH6) accounted for 1.74% of the variants in the total population tested. Whereas BRCA2 mutations have been associated with more aggressive disease and poor clinical outcomes, the prognostic implications of other DNA damage repair genes are not as established. BRCA1 and PALB2 are mutated in less than 1% of castrate-resistant prostate cancer.

Mutations in ATM and CDK12 are more common and are each present in 3% to 6% of cases. Mutation status of BRCA1/BRCA2 and ATM is also associated with grade reclassification among men undergoing active surveillance. Other DNA damage repair genes that are being tested in advanced prostate cancer include FANCA, RAD51D, and CHEK2.

Shen and Rubin also address this issue in some detail.

3.4 METABOLICS

The cell metabolic factors can come to dominate many cancers. Cancer cells can manage to survive and prosper in what would normally be a stressed environment. As Ahn et al have noted:

For decades, evidence has suggested that metabolic syndrome and its components are associated with increased development and progression of aggressive PCa. For incidental PCa, studies have reported no or an inverse relationship with metabolic syndrome. Recently…reported that metabolic syndrome and its components may hinder the diagnosis of low-stage PCa by a

mechanism that reduces serum prostate-specific antigen (PSA) level. Thus, the observed inverse relationship between metabolic syndrome and low-stage PCa may be the result of diagnostic bias, rather than the underlying biology associated with its development. Androgen deprivation therapy (ADT) plays an important role in the treatment of advanced PCa, either as monotherapy or combined with radical prostatectomy (RP) or radiation therapy (RT).

ADT may be utilized in all stages of PCa and confers increased survival in advanced stages of the disease. However, ADT is associated with a wide range of adverse effects and reduced quality of life. In terms of metabolic syndrome, reduced level of circulating testosterone induces increased circulating insulin level, insulin resistance, changes in body composition, fatigue, sexual dysfunction, decreased bone mineral density, hyperlipidemia, and acute coronary syndrome. These side effects may compromise OS outcomes.

Insulin promotes local androgen synthesis by PCa cells; this is considered one of the mechanisms in the development of castration-resistance. In a retrospective study…metabolic syndrome was associated with a shorter time to PSA progression and inferior OS in patients with PCa receiving ADT.

Therefore, if metformin can exert positive effects on hyperinsulinemia and metabolic syndrome, it may be potentially utilized as an adjunctive treatment in reducing the risk of castration resistance in patients on long-term ADT.

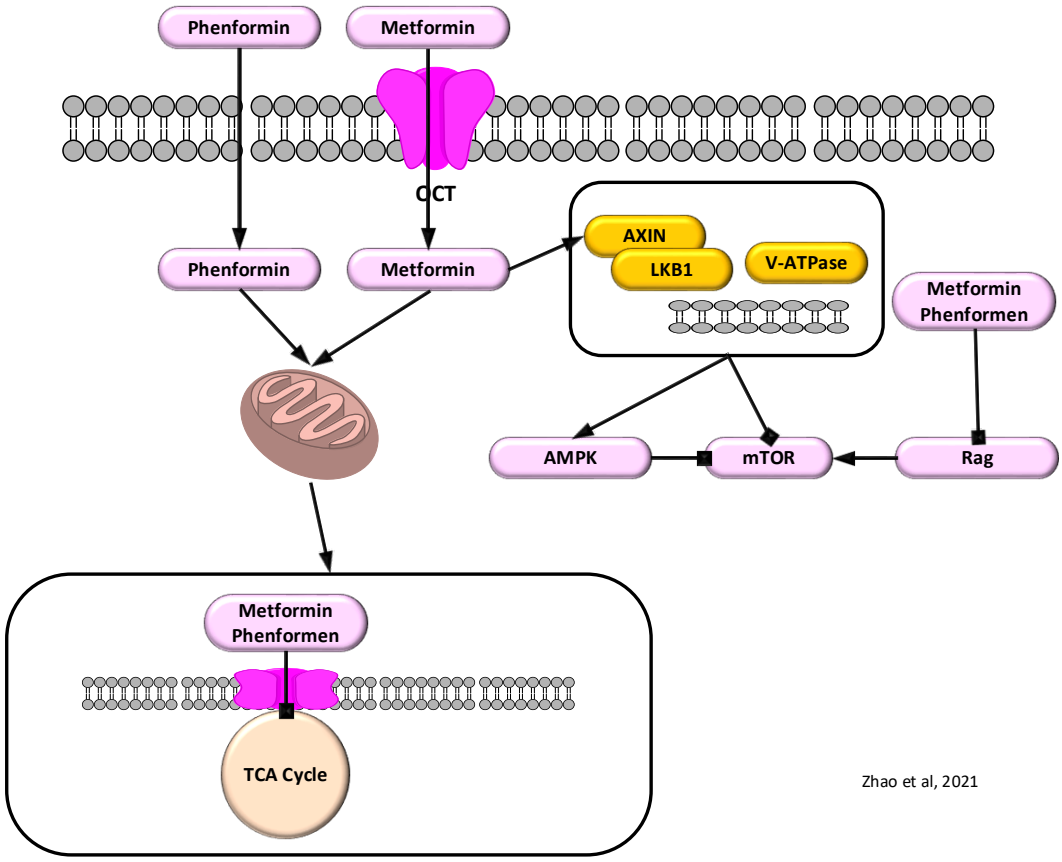
Overall PCa has extensive complexities. As is well known, many patients have an indolent form of PCa and there are highly aggressive forms as well, albeit much fewer in number.

4 BIGUANIDES AND CANCER

We now examine the use of biguanides in cancers, and then focusing on PCa. The mechanisms seem fairly well understood but wide scale clinical evidence is still limited.

4.1 BASIC PRINCIPLES

From the work of Zhao et al we have demonstrated below the metabolic issues related to metformin and its related phenformin, a stronger molecule.



Ahn et al have noted:

Metformin, an oral biguanide used for first-line treatment of type 2 diabetes mellitus, has attracted attention for its anti-proliferative and anti-cancer effects in several solid tumors, including prostate cancer (PCa).

Liver kinase B1 (LKB1) and adenosine monophosphate-activated protein kinase (AMPK) activation, inhibition of the mammalian target of rapamycin (mTOR) activity and protein synthesis, induction of apoptosis and autophagy by p53 and p21, and decreased blood insulin level have been suggested as direct anti-cancer mechanisms of metformin.

Research has shown that PCa development and progression are associated with metabolic syndrome and its components.

Therefore, reduction in the risk of PCa and improvement in survival in metformin users may be the results of the direct anti-cancer mechanisms of the drug or the secondary effects from improvement of metabolic syndrome.

In contrast, some research has suggested that there is no association between metformin use and PCa incidence or survival. In this comprehensive review, we summarize updated evidence on the relationship between metformin use and oncological effects in patients with PCa. We also highlight ongoing clinical trials evaluating metformin as an adjuvant therapy in novel drug combinations in various disease settings.

4.2 MIRNAS

miRNAs a small RNAs, 22 nucleotides, which can have a significant impact on the expression of genes and translation into active proteins[15]. Alimoradi et al have recently presented a review paper on metformin and miRNA control in various cancers. They note:

MiR-708-5p is a circulating miR which acts as a tumor suppressor by targeting an endoplasmic reticulum (ER) protein neuronatin (NNAT), causing a decrease in intracellular calcium.

Metformin treatment dramatically upregulates miR-708-5p expression resulting in inhibition of calcium uptake by ER.

Decline in intracellular calcium causes ER stress and as a result, cell apoptosis is activated. Thus, NNAT is identified as a novel target of metformin in induction of apoptosis for prostate cancer cells

We have previously discussed miRNAs in PCa but this observation may be useful as an extension. It is not clear just how effective this process is but it is intriguing and worth following.

4.3 RESULTS

We can summarize several recent results. Liu et al (2018) have noted:

1. *Metformin inhibits castration-induced inflammatory infiltration*

2. *Metformin inhibits inflammatory infiltration by targeting the COX2/PGE2 axis*

3. *Metformin represses macrophage migration/recruitment by prostate cancer cells*

Wang et al have similarly noted:

[15] https://www.researchgate.net/publication/338684968_miRNAS_REDUX

1. *Expression of TNF-α and NF-κB-P65 was upregulated, while E-cadherin expression was downregulated in PCa specimens with a high Gleason score*

2. *Metformin inhibited TNF-α-induced epithelial–mesenchymal transition in PC3 cells*

3. *Metformin suppressed TNF-α-induced PC3 cell migration and invasion capacity*

4. *Metformin inactivated the NF-κB signaling pathway in PC3 cells*

Wang et al continue:

The present study demonstrated that the expression of TNF-α and NF-κB-P65 was significantly upregulated in PCa tissues and positively correlated with the Gleason score, while E-cadherin expression was significantly downregulated in PCa tissues and negatively correlated with the Gleason score. Moreover, metformin effectively inhibited the TNF-α-induced migration ability and invasion activity of PC3 cells. Furthermore, metformin might suppress the TNF-α-induced EMT process by inactivating the NF-κB signaling pathway.

Mortality in PCa patients is mainly caused by tumor metastasis.

Recently, it was demonstrated that chronic inflammation plays a key role in PCa metastasis, which is a major challenge during PCa therapy. It is well known that inflammatory mediators, such as ***TNF-α, TGF-β and IL- 6***[16]*, are involved in the migration, invasion and metastasis of malignant cells. TNF-α is a major proinflammatory cytokine that participates in a series of biological activities, including inflammation, cell proliferation, cell differentiation and apoptosis. TNF-α is able to activate the canonical NF-κB pathway in various cell types.*

When cells are stimulated by TNF-α, IKK-β is first activated, and then the NF-κB inhibitor IκBα is phosphorylated and rapidly degraded. This allows the NF-κB heterodimer to translocate into the nucleus and activate the expression of numerous downstream target genes implicated in angiogenesis, the immune response, cell proliferation and cell apoptosis. The EMT process can be induced by various growth factors and cytokines, which are produced by cell activation (30-32). We administered TNF-α to prostate cancer PC3 cells to investigate the effect of TNF-α on EMT characteristics in prostate cancer cells.

Our results demonstrated that TNF-α-treated PC3 cells showed a significant increase in cell migration ability and invasion activity, suggesting a possible role of TNF-α in the migration behavior of prostate cancer cells. A hallmark of EMT is the loss of the cell adhesion molecule E-cadherin. Our results showed that TNF-α decreased the expression of E-cadherin and increased the expression of N-cadherin and Vimentin, but these changes were reversed by BAY11-7082, an

[16] https://www.researchgate.net/publication/340607207_IL-6_COVID-19_Cytokine_Storms_and_Galen It should be noted that we saw the impact of IL-6 in COVID and that recently NRJM reported that our recommendation in early 2020 has significant effectiveness and efficacy, https://www.nejm.org/doi/full/10.1056/NEJMoa2100433?query=featured_home , Interleukin-6 Receptor Antagonists in Critically Ill Patients with Covid-19, April 22, 2021.

inhibitor of NF-κB. These results demonstrated that TNF-α might promote the EMT process in prostate cancer cells via the NF-κB signaling pathway.

In a 2013 paper by Margel et al the authors had noted:

Increased cumulative duration of metformin exposure after PC diagnosis was associated with decreases in both all-cause and PC-specific mortality among diabetic men.

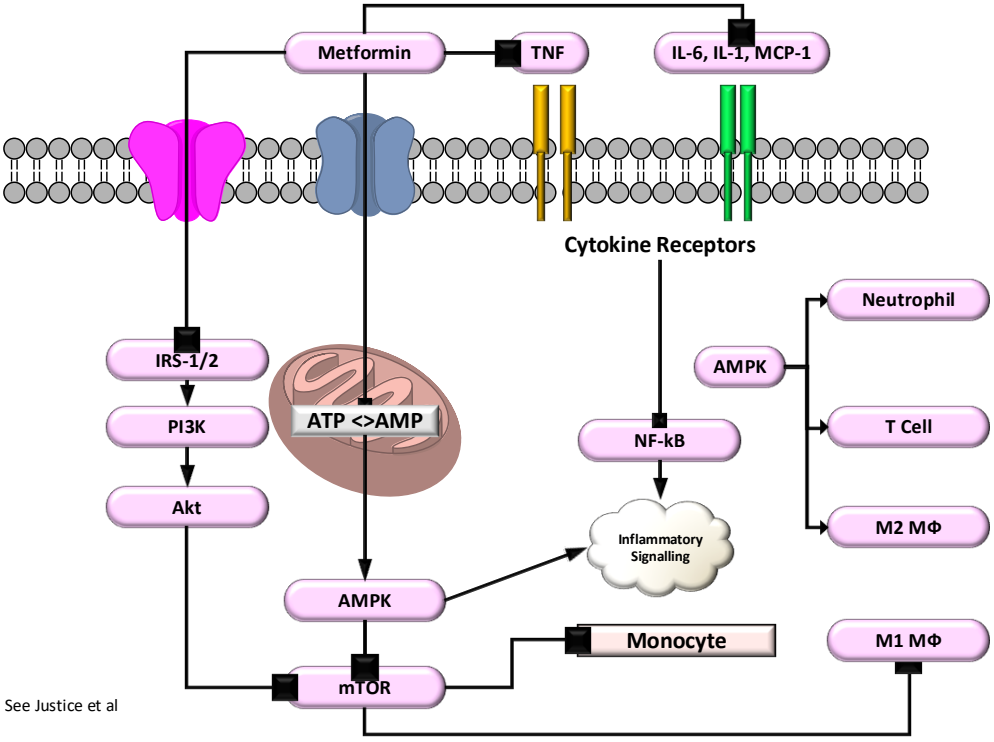
Admittedly the data was limited but clearly after almost a decade of ongoing trials the results appear to have gotten stronger rather than less so.

5 IMMUNE SYSTEM ISSUES

Immunotherapy has been an explosive field over the past decade[17]. In this section we highlight the issues related to PCa and metformin.

5.1 BASIC PRINCIPLES

Justice et al demonstrate the impact of metformin and various elements of the immune system. The figure below relies on that work as modified. The intent is to demonstrate the impact that metformin has a various immune cells.



Justice et al also note regarding the above:

Metformin alleviates chronic proinflammatory immune signaling and restores immune response. Metformin's cellular mechanisms include weak inhibition of complex I of the mitochondrial electron transport chain, activation of the energy sensor AMP-activated protein kinase, inhibition of the hetero-multimeric protein kinase mTORC1, and suppression of elevated

[17] See https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach This is a 2017 view of immunology and immunotherapy as a Precis and introduction. As with everything in this field it has seen dramatic changes year by year.

proinflammatory cytokines production. Converging evidence also implicates the gut microbiome which further alleviates inflammation and phagosome-lysosome fusion which induces phagocytosis of neutrophils to reduce pathogen burden. The collective result is a dampened broad proinflammatory cytokine signaling and improved immune cell activation

5.2 T CELLS

T cells are the powerhouse of the adaptive immune system. We summarize the key ones here. It is important to note, however, that the TME can create a protective shield around the PCa core that the T cells may not be able to penetrate. However, there is a body of research indicating the possibilities. The following is a summary based on Abbas et al.

T Cell Type	
Th1	Subset of CD4+ helper T cells that secrete a particular set of cytokines, including **IFN-γ, and whose principal function is to stimulate phagocyte-mediated defense against infections,** especially with intracellular microbes.
Th2	Functional subset of CD4+ helper T cells that secrete a particular set of cytokines, including **IL-4, IL-5, and IL-3 and whose principal function is to stimulate IgE and eosinophil/mast cell**–mediated immune reactions.
Th17	Functional subset of CD4+ helper T cells that secrete a particular set of inflammatory cytokines, including **IL-17, which are protective against bacterial and fungal infections and also mediate inflammatory reactions in autoimmune and other inflammatory diseases.**
Treg	Population of T cells that regulates the activation of other T cells and is necessary to maintain peripheral tolerance to self-antigens. Most regulatory T cells are CD4+ and constitutively express CD25, the α chain of the IL-2 receptor, and the transcription factor FoxP3.
T FH	Heterogeneous subset of CD4+ helper T cells present within lymphoid follicles that are critical in providing signals to B cells in the germinal center reaction. **TFH cells express CXCR5, ICOS, IL-21, and Bcl-6**
TIL CTL	Type of T lymphocyte whose major effector function is to recognize and kill host cells infected with viruses or other intracellular microbes. **CTLs usually express CD8 and recognize microbial peptides displayed by class I MHC molecules.** CTL killing of infected cells involves the release of cytoplasmic granules whose contents include enzymes that initiate apoptosis of the infected cell and proteins that facilitate entry of these enzymes into the target cells.

T Cell Type	
TRN[18]	Tissue-resident memory T cells (TRM) provide immune defence against local infection and can inhibit cancer progression. However, it is unclear to what extent chronic inflammation impacts TRM activation and how the immune pressure exerted by TRM affects developing tumours in humans

As Kurelac et have noted:

Metformin inhibits immunosuppressive responses by boosting cytotoxic T-lymphocyte functions.

Cancer cells are able to suppress the cytotoxic effects of lymphocytes by various mechanisms, the most well-known being the overexpression of programmed death ligand 1 (PD-L1), which causes cytotoxic T-cell exhaustion, resulting in immunosuppression and cancer cell survival. On the other hand, tumors often harbor high numbers of protumorigenic regulatory T-cells (Treg), which support tumor growth by promoting wound-healing-like signals. The current literature generally agrees that metformin boosts anti-tumor adaptive immune response.

Increased tumor infiltrating lymphocyte (TIL) abundance and enhanced cytotoxic T-cell functions were described both in primary tumor and in metastatic experimental settings upon metformin treatment. *Eikawa was first to show that, in contrast to the anti-survival effect ascribed to metformin regarding cancer cell viability, metformin treatment may protect TILs from apoptosis.*

Moreover, metformin was shown to increase TIL multifunctionality (triple inflammatory cytokine production: IL-2, TNF-α, IFN-γ), regardless of their PD-L1 status, a phenomenon which could be abrogated by the AMPK inhibitor compound C.

Since metformin should decrease mitochondrial respiration simultaneously in T-lymphocytes and cancer cells, leaving glycolysis as the common metabolic engine in both cell types, and thus promoting competition for glucose, it is intuitive to hypothesize such avidity for sugar would lead to glucose shortage in the TME and eventually block cytotoxic T-cell effector function.

Nevertheless, the study which predominantly focused on RLmale1 tumors in Balb/c mice, showed metformin to exhibit anti-tumorigenic effects via direct action on CD8+ TILs, as it reduced their exhaustion, raising the question about how metformin promotes cytotoxic T-cell phenotype. One possible explanation comes from a study of cancer progression in obese models which suggested that metformin in combination …with targeting PlGF/VEGF1R pathway allows a higher influx of cytotoxic T cells into the tumor site due to increased perfusion.

This hypothesis was drawn also for the cytotoxic T and NK cells in the context of pancreatic cancer where high T-cell numbers in metformin treated masses have been associated with improved vascularization and reduced dysplasia.

[18] https://www.biorxiv.org/content/10.1101/2021.04.20.440373v1?rss=1 Weeden et al, Early immune pressure imposed by tissue resident memory T cells sculpts tumour evolution in non-small cell lung cancer.

Interestingly, hypoxia was shown to reduce IFN-γ expression and T-cell cytolytic activity against cancer cells, whereas elevating intracellular oxygen concentration by metformin resulted in increased T-cell activation, suggesting that hypoxic signalling modulates T-cell phenotype regardless of the tumor perfusion status.

Moreover, metformin was found to downregulate HIF1 in ovarian cancer myeloid-derived suppressor cells (MDSCs), decreasing their immunosuppressive activity and improving cytotoxic T-cell functions, pointing out to an indirect effect of hypoxia on the anti-tumor Tcell activity. Of note, a reduced MDSCs immunosuppressive action on T-cells was observed also upon treatment with biguanidine phenformin.

Another mechanism through which metformin promotes cytotoxic T-cell phenotype was recently uncovered …in a detailed and convincing set of experiments, explain that high CD8+ T-cell mediated cytotoxic activity in metformin-treated 4T1 breast tumors in BALB/c is due to downregulation of PD-L1 in cancer cells.

In particular, AMPK activated by metformin caused endoplasmic-reticulum associated degradation of PD-L1, prevented its processing to Golgi and decreased PD-L1 localization on the cancer cell membrane, eventually boosting the effect of cytotoxic T lymphocytes.

… metformin did not have an anti-tumorigenic effect in immunodeficient SCID mice, which prompted the authors to attribute the anti-tumorigenic properties of the drug mainly to T-cell activity. However, their findings should not be generalized, since many studies in nude immunodeficient mice concur on the anti-tumorigenic effect of metformin. Different outcomes could be due to the fact that …early response to the drug (10–20 days). Moreover, diverse metformin effects are most likely dependent on the oncogene driving the transformation, as it was observed, for example, that its antitumorigenic potential is modulated based on whether cancer cell transformation is associated or not with an inflammatory signature.

Apart from promoting cytotoxic T-cell functions, it has been reported that the immune cell-mediated anti-tumorigenic effects of metformin may be exerted also by downregulating pro-tumorigenic lymphocytes.

In particular, …in a study on orthotopic hepatocellular carcinoma, showed that metformin prevents differentiation of a specific subtype of T helper cells (Th1 and Th17) producing wound-healing-associated cytokine IL-22, which eventually leads to reduction of hepatocellular cancer cell growth in BALB/c livers. Moreover, metformin was reported to prevent Treg infiltration into tumors, via mammalian Target of rapamycin complex (mTORC1) inhibition and subsequent Foxp3 downregulation, normally required for Treg differentiation

Thus, there seems to be clear evidence that T cells along with metformin play a significant role in tumor suppression. More importantly is the fact that metformin enables penetration into the TME. In that regard the authors note:

Metformin may exert protective effects on the endothelial TME component but inhibits proangiogenic signals in cancer cells *The vascular architecture of a tumor is a consequence of multiple angiogenic signals deriving from either cancer cells, or other cells in the TME, including endothelial cells themselves. Thus, here we distinguish direct and indirect effects of metformin on tumor angiogenesis. Most in vitro and in vivo studies have reported that metformin affects tumor angiogenesis indirectly, by modulating cancer cell-mediated angiogenic signals.*

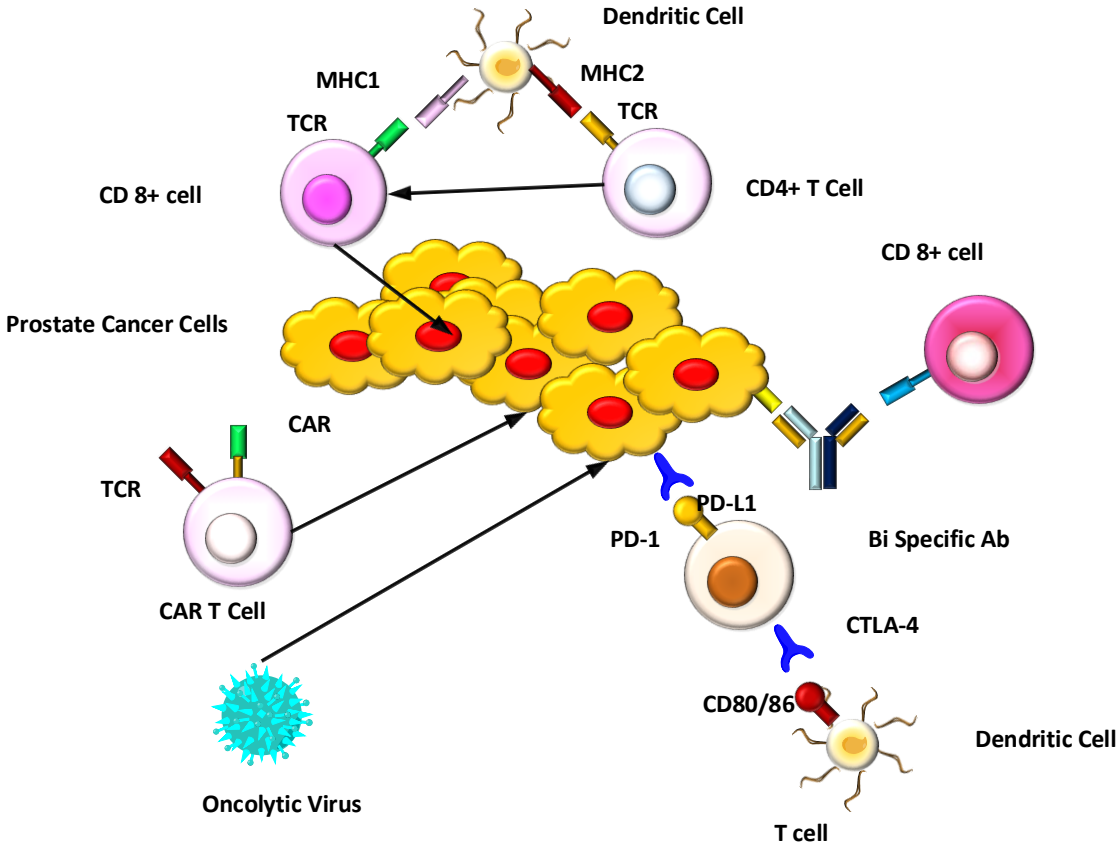
Metformin has mostly been associated with a decrease in hypoxia-inducible factor 1 alpha (HIF-1α) stability in cancer cells, reducing the expression of HIF1-targeted genes, including VEGFA, and thus resulting in slow-growing tumors, often characterized by smaller tumor vessel size and reduced microvessel density. Similarly, we have shown that targeting mitochondrial CI specifically in cancer cells prevents HIF1 activation, and results in immature vasculature

Thus, metformin appears to have multiple roles which can be utilized in a complex set of therapeutics both adjuvant and neo-adjuvant.

5.3 IMMUNOTHERAPY OPTIONS

Let us begin with a graphic which delineates the multiple lines of attack using the immune system. Some have been tried in PCa but only Sipuleucel has been approved and in use. It relies on dendritic cell modification. There are a multiplicity of PD-1 and CTLA-4 immunotherapeutics yet none have yet demonstrated an acceptable effectiveness. There are studies showing OS and PFS benefits but frankly they seem modest at the current time.

For each of the possible points of attack below we now have a therapeutic to approach it. The critical factor is that the immune cells must reach the tumor cell often going through the mine field of the TME.



Note that the use of bi-specific antibodies is just one of many added poly specific approaches[19].

5.4 METFORMIN AND THE IMMUNE PROCESS

Let us now examine the impact of metformin on the immune system. Liu et al (2018) have noted:

Inflammatory infiltration has been considered as a double-edged sword in tumor biology because it can either aid or fight tumors depending on specific tumor microenvironment.

Although some studies found that in certain circumstances inflammatory infiltration could be inhibitive in tumor progression by maintaining organ homeostasis and ensuring stable tissue structure, more evidence supports the conclusion that chronic inflammation contributes to tumor initiation, metastasis, and progression. A meta-analysis …found that 15% cancers could be directly attributed to the infection of viruses, bacteria, and parasites; and individuals with chronic inflammation generally have high cancer incidence. Furthermore, the number of infiltrated inflammatory cells has been suggested as a hallmark of a tumor.

Our tissue microarray data showed that the numbers of TAM infiltrated in the TME were positively correlated with Gleason scores, suggesting TAM infiltration is also associated with the

[19] https://www.researchgate.net/publication/346245151_Poly-specific_Antibodies

malignancy of prostate cancer, and these findings were in line with the number of TAMs and the severity of tumors in our mouse model. Due to their plasticity and flexibility, monocytes differentiate into macrophages with distinct phenotypes depending on their microenvironment.

There are ***two main subtypes of macrophages****.*

The ***M1-like macrophages promote Th1 response with strong microbicidal and tumoricidal activity,*** *and the*

M2-like macrophages usually promote Th2 response, tissue remodeling, immune tolerance, and tumor progression*.*

Under certain circumstances, the subtypes are interchangeable depending on their local microenvironment.

In addition, ***M2-like macrophages can be further divided into four subgroups (M2a, M2b, M2c, and M2d).*** *More recent evidence suggests that TAMs and M2d subtype share more characteristics such as promoting tumor growth, metastasis, and angiogenesis. Similarly… multiple cell surface markers specific for M2-like macrophage have been identified. However, not all markers were found on the surface of every M2-like cell.*

This finding is consistent with that not all M2-like markers are necessarily required for every cell. IHC staining of the consecutive sections of human lymphoma (positive control) using three markers (CD68, CD163, and CD204) commonly used for identification of M2-like macrophages showed that these markers were not completely colocated . In addition, by using immunofluorescent double and triple labeled staining, we found that most of the macrophages express either two (CD163 and CD204) or three (CD68, CD163, and CD204) markers simultaneously, although a few cells only expressed one of them.

Although the intensities of immunostaining of these markers varied noticeably in our results, the overall intensities of these markers were significantly reduced in the metformin-treated group, indicating that metformin is capable of inhibiting the recruitment of TAMs in the TME.

Multiple lines of evidence imply that infiltrated TAMs interact with tumor cells and TAMs play crucial roles in most, if not all, processes of tumor development.

Activation of transcription factors such as NF-kB, STAT3, and HIF1a in tumor cells by either inflammation or infection leads to the secretion of wide spectrum factors including cytokines, chemokines, and prostaglandins.

These factors collectively result in the recruitment of TAMs, and inflammatory mediators secreted by the TAMs lead to further recruitment of more TAMs. Through some ill-defined mechanisms, the TAMs enhance different processes in cancer initiation and development including proliferation, survival, EMT, angiogenesis, migration, invasion and metastasis, as well as the development of resistance to various treatments.

In this study, we demonstrated that metformin is capable of inhibiting TAM recruitment both in vivo and in vitro and reduced TAM recruitment concurrently accompanied by less tumor cell metastasis.

Elevated levels of COX2 in prostate cancer cells were seen in both the TRAMP model and human prostate adenocarcinoma. We demonstrated in this study that metformin treatment not only downregulated COX2 and its product PGE2 in prostate cancer cells but also inhibited the recruitment of TAMs. On the other hand, exogenously added PGE2 was able to counteract metformin-mediated downregulation of COX2 and rescue the recruitment of TAMs as well as cancer cell migration, suggesting PGE2 plays a crucial role in TAM recruitment.

Of note, fewer TAMs in the TME were accompanied by reduced cytokines and chemokines. These lines of evidence are consistent with the inhibitory role of metformin in prostate cancer cell migration and macrophage recruitment.

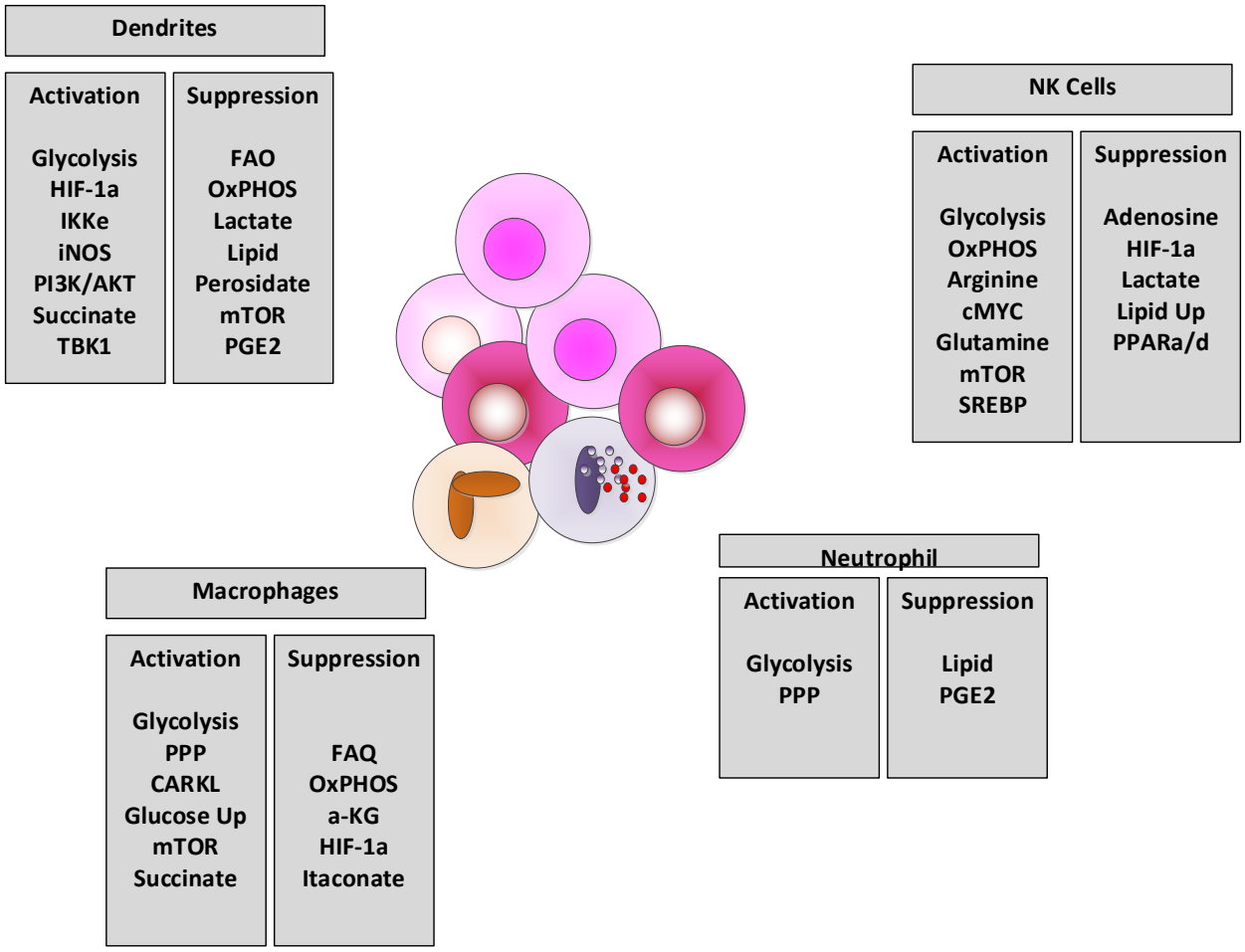
Therefore, we conclude that the inhibitory effect of metformin on the recruitment of TAMs and cancer cell migration is at least in part by directly downregulating COX2, which subsequently reduced the levels of PEG2.

In addition, by using PC-3 and DU145 prostate cancer cell lines, we demonstrated that metformin might have some direct inhibitory effects on proliferation and cell cycle, as well as acceleration of the apoptosis. Moreover, we found that metformin could also inhibit the functions of macrophages, such as producing cytokines IL6 and TNFa induced by LPS.

5.5 INNATE IMMUNE RESPONSE

The innate immune system has frequently been dismissed with emphasis on the more complex adaptive system. However the innate does have a set of cells that perform vital and at times defensive actions regarding new lesions. The chart below is a graphic of how this may act both positively and negatively for many of the key innate cells. In our experience the NK cells can be highly effective players in asserting attacks on cancers cells. We have seen this in the CIK cells in the cases of MDS[20]. The figure below is derived from Talty and Olino.

[20] https://www.researchgate.net/publication/334959399_Immunotherapy_Possible_Directions



The authors then note regarding metformin in the context of trials:

Metformin

AMPK activation; mitochondrial glycerophosphate dehydrogenase inactivation

Anti-PD-1 plus/minus metformin in advanced melanoma, renal cell carcinoma, non-small-cell lung carcinoma, hepatocellular carcinoma, urothelial cancer, or head and neck squamous cell carcinoma (NCT04114136)

Platinum chemotherapy and metformin plus/minus fasting mimicking diet to target the metabolic vulnerabilities of LKB1-inactive lung adenocarcinoma (NCT03709147)

Anti-PD-1 with or without metformin in treating participants with head and neck squamous cell carcinoma to evaluate alterations in T cell and TAM polarization (NCT03618654)

where they state:

Several ongoing clinical trials focus on combining metabolism-targeted agents with immunotherapy treatments….

Many of these trials exploit the use of previously approved drugs such as metformin and rosiglitazone which are used in the treatment of diabetes and alter downstream metabolic pathways.

For example, metformin decreases peripheral insulin resistance by inhibiting mitochondrial respiration and activating AMPK. Activated AMPK inhibits metabolic processes such as gluconeogenesis and lipogenesis and stimulates glucose uptake and fatty acid oxidation thus affecting additional pathways tied in with immunometabolism.

Metformin can also target mTOR, insulin-like growth factor, and mitogen-activated protein kinase (MAPK) pathways. In various preclinical studies, metformin has been shown to potentiate antitumor immunity more directly by promoting STING and Hippo signaling, PD-L1 degradation, and a reduction in tumor hypoxia. Rosiglitazone activates PPARγ and has had similar preclinical results.

None are prostate specific.

6 OBSERVATIONS

We can now make several observations that would require additional insight. However, in the six years since our first observations, the use of metformin in PCa has been significantly strengthened. As we will note, some have even considered its use as a means to longevity. One must be cautious, however, when asserting excess capabilities until adequate trial data is presented. Nevertheless, the evidence to date is truly compelling.

6.1 ADJUVANT OR NEO-ADJUVANT OR PREVENTATIVE

The use of therapeutics, namely their timing, has come under some significant scrutiny. The classic approach is to perform surgery, then wait, then use a therapeutic. However, with immunotherpeutics there is a "learning phase" and then a "seek and destroy". Learning can be enhanced by the presence of many cancer cells. This neo-adjuvant therapy has become useful in this context.

Thus when examining metformin, it appears that its use in a neo-adjuvant application is warranted at least in several controlled trials.

6.2 CLINICAL TRIALS

Many of the current trials have focused on second generation AR inhibitors. As Zamangni et al have noted regarding them:

Enzalutamide *has greater affinity for AR-LBD than bicalutamide, with an additional strong effect on AR mutant W741C. In the PROSPER phase III trial (NCT02003924) enzalutamide-based therapy led to a striking 71% lower risk of metastasis and death, compared to placebo, among men with nonmetastatic CRPC).*

Apalutamide *is an AR targeted antiandrogen with a chemical structure very similar to enzalutamide, but characterized by a better affinity to ARLBD. It lacks agonist activity, in contrast with bicalutamide, and inhibits nuclear translocation and DNA binding. Moreover, apalutamide showed less blood-brain barrier penetration in murine xenograft models of CRPC than enzalutamide, which might lead to less seizure than enzalutamide-based therapies. On the basis of the SPARTAN trial (NCT0946204), apalutamide was FDA-approved in 2018 for patients with nonmetastatic CRPC…*

Darolutamide *is an antiandrogen with higher potency and efficacy toward ARLBD compared to enzalutamide and apalutamide, but very similar mechanism and pharmacology. The results of the phase III ARAMIS trials (NCT02200614) have just been published, suggesting this new AR inhibitor as an alternative option for nonmetastatic CRPC patients. ARAMIS results are indeed in line with those of the SPARTAN and the PROSPER trials, involving apalutamide and enzalutamide, respectively. Since darolutamide has a different molecule structure, it has to be taken into account that it may be related to different adverse effects. Thus, further observations on real-world data are needed to better define the clinical niche for each compound*

Saad et al have provided a recent review of various clinical trials on androgen receptor inhibitors for nmCRPC. They conclude:

While the definition of nmCRPC is well established, the advent of next-generation imaging techniques capable of detecting hitherto undetectable oligometastatic disease in patients with nmCRPC has fostered debate on the criteria that inform the management of these patients. However, despite these developments, published consensus statements have maintained that the absence of metastases on conventional imaging suffices to guide such therapeutic decisions. In addition, the prolonged metastasis-free survival and recently reported positive overall survival outcomes of the three second-generation androgen receptor inhibitors have provided further evidence for the early use of these agents in patients with nmCRPC in order to delay metastases and prolong survival.

Here, we discuss the benefit–risk profiles of apalutamide, enzalutamide, and darolutamide based on the data available from their pivotal clinical trials in patients with nmCRPC …

Clinical development in the field of nmCRPC is evolving rapidly. The second-generation ARIs, ***apalutamide, enzalutamide, and darolutamide****, have revolutionized the treatment landscape for nmCRPC.*

All three ARIs have demonstrated significant prolongation of MFS, and the recently reported final analyses of the SPARTAN, PROSPER, and ARAMIS trials indicate a significant OS benefit in patients with nmCRPC. Although second-generation ARIs have acceptable tolerability and maintain quality of life in patients with nonmetastatic disease, their individual safety profiles and potential for drug–drug interactions with concomitant medications should be considered. The benefit–risk profile of ARIs in patients with nmCRPC is an important clinical consideration and therapies that do not compound ADTrelated AEs or contribute to additional therapeutic burden due to drug–drug interactions may be preferred.

In summary, while delaying the onset of metastasis and ultimately prolonging survival represents the central objective of pharmacotherapy, appropriate treatment of patients with nmCRPC must strike an individualized balance between clinical benefit and potential risk

Kim et al also provides an excellent review of trials and recent therapeutics.

6.3 MULTI-THERAPEUTICS

Combination therapies or "multi-therapeutics" are often more effective and efficacious than a single therapy or sequential use of the same therapeutics. Recent experience with CTLA4 and PD-1 immunotherapeutics has shown some significant improvement. As Xie et al have noted:

We explored whether the anti-prostate cancer (PC) activity of the androgen receptor axis-targeted agents (ARATs) abiraterone and enzalutamide is enhanced by metformin. Using complementary biological and molecular approaches, we determined the associated underlying mechanisms in pre-clinical androgen-sensitive PC models. ARATs increased androgren

receptors (ARs) in LNCaP and AR/ARv7 (AR variant) in VCaP cells, inhibited cell proliferation in both, and induced poly(ADP-ribose) polymerase-1 (PARP-1) cleavage and death in VCaP but not LNCaP cells[21]. Metformin decreased AR and ARv7 expression and induced cleaved PARP-1-associated death in both cell lines.

Metformin with abiraterone or enzalutamide decreased AR and ARv7 expression showed greater inhibition of cell proliferation and greater induction of cell death than single agent treatments. Combination treatments led to increased cleaved PARP-1 and enhanced PARP-1 activity manifested by increases in poly(ADP-ribose) (PAR) and nuclear accumulation of apoptosis inducing factor (AIF).

Enhanced annexin V staining occurred in LNCaP cells only with metformin/ARAT combinations, but no caspase 3 recruitment occurred in either cell line. Finally, metformin and metformin/ARAT combinations increased lysosomal permeability resulting in cathepsin G-mediated PARP-1 cleavage and cell death. In conclusion, metformin enhances the efficacy of abiraterone and enzalutamide via two PARP-1-dependent, caspase 3-independent pathways, providing a rationale to evaluate these combinations in castration-sensitive PC.

6.4 TME AND CANCER CELL ATTACK

The greatest issue we have seen in examining cancers is the impact of the TME, the stroma, the fibroblasts, the macrophages, and all the protective and supporting elements. Metformin is a small and readily transported molecule and it appears accessible to the malignant cells even in a dense TME.

6.5 BROAD BASED USE OF METFORMIN

In a recent paper by Justice et al the authors set out a broad perspective for the significant use of metformin beyond just T2D and the PCa application contained herein. They conclude:

In conclusion, metformin is an attractive tool or probe for clinical trials targeting aging and to improve host immune defense and resilience in COVID-19 and infectious disease. Its immunoprotective effects are hypothesized to (i) lessen severity of unfavorable health outcomes or death in the event of exposure to infectious disease; (ii) delay or prevent long-term chronic diseases or conditions which can stem from acute challenges or viral infections; and (iii) bolster immune response to vaccine. However, this hypothesis has yet to be rigorously tested.

It is time for definitive geroscience trials of not only metformin but other promising geroprotective interventions like caloric restriction, mTOR inhibitors, and senolytics. Trials informed by geroscience will offer opportunity to investigate intervention effects on vaccine response and resilience to age-related chronic diseases and geriatric syndromes and provides a unique opportunity to advance study of host-immune defense with implication for recovery from the current pandemic and unforeseen future challenges.

[21] https://www.researchgate.net/publication/313900832_PARP_and_Prostate_Cancer This discusses the use of PARP on PCa.

Namely, they assert that metformin has broad ranging benefits beyond what we have discussed herein. We have seen this stated elsewhere and are always concerned to such a far reaching claim. Yet there seems to be both logical and clinical basis regarding the application in PCa.

7 REFERENCES

1. Abbas et al, Basic Immunology, 4th Ed, Elsevier, 2014
2. Ahn et al, Current Status and Application of Metformin for Prostate Cancer: A Comprehensive Review, Inter Jrl Mol Sci, 12 Nov 2020
3. Akoto and Saini, Role of Exosomes in Prostate Cancer Metastasis, Molecular Sciences, 29 March 2021
4. Alimoradi et al, How metformin affects various malignancies by means of microRNAs: a brief review, Cancer Cell Int (2021) 21:207
5. Allott, et al, Postoperative statin use and risk of biochemical recurrence following radical prostatectomy: results from the Shared Equal Access Regional Cancer Hospital (SEARCH) database, BJU International, Volume 114, Issue 5, pages 661–666, November 2014
6. Baldo, P., et al, mTOR Pathway and mTOR inhibitors as Agents for Cancer Therapy, 2008, Curr Can Drug Targets, pp 647-668.
7. Bass, A., et al, Complete Polarization of Single Intestinal Epithelial Cells upon Activation of LKB1 by STRAD, Cell, Vol. 116, 457–466, February 6, 2004
8. Bauer, A., C. Stratakis, The lentiginoses: cutaneous markers of systemic disease and a window to new aspects of tumourigenesis, J Med Genet 2005;42:801–810. doi: 10.1136
9. Bonollo et al, The Role of Cancer-Associated Fibroblasts in Prostate Cancer Tumorigenesis, Cancers, 13 July 2020
10. Cantley et al, Signal Transduction, CSHL Press, 2014
11. Chan K, at al. The statins as anticancer agents. Clin Cancer Res 2003; 9: 10–19.\
12. Chang et al, Overexpression of the recently identified oncogene REDD1 correlates with tumor progression and is an independent unfavorable prognostic factor for ovarian carcinoma. Diagn Pathol 13, 87 (2018).
13. Danzig, et al, Synergism between metformin and statins in modifying the risk of biochemical recurrence following radical prostatectomy in men with diabetes, Prostate Cancer and Prostatic Disease (2015) 18, 63–68
14. Delma, Three May Be Better Than Two: A Proposal for Metformin Addition to PI3K/Akt Inhibitor-antiandrogen Combination in Castration-resistant Prostate Cancer, Cureus 10(10), 2018
15. Easton, J., P. Houghton, mTOR and Cancer Therapy, Oncogene, 2006, pp 6436-6446.
16. Epstein, Biopsy Interpretation of the Prostate, 6th Ed, Wolters Kluwer, 2021
17. Fizazi et al, Nonmetastatic, Castration-Resistant Prostate Cancer and Survival with Darolutamide, NEJM, 2020;383:1040-9
18. Hua et al, Targeting mTOR for cancer therapy, Journal of Hematology & Oncology (2019) 12:71

19. Justice et al, A geroscience perspective on immune resilience and infectious diseases: a potential case for metformin, GeroScience, 2020
20. Kim et al, Current Status and Future Perspectives of Androgen Receptor Inhibition Therapy for Prostate Cancer: A Comprehensive Review, Biomolecules, 2021, 11,492
21. Kinkaide, C., et al, Targeting Akt/mTOR and ERK MAPK Signalling Inhibits Hormone Refractory Prostate Cancer in Preclinical Mouse Model, Jrl Clin Inv 2008, 99 3051-3064.
22. Knura et al, The Influence of Anti-Diabetic Drugs on Prostate Cancer, Cancers, 2021, 13, 1827
23. Kokal et al, Mechanisms of Androgen Receptor Agonist- and Antagonist-Mediated Cellular Senescence in Prostate Cancer, Cancers, 8 July 2020
24. Kurelac et al, he multifaceted effects of metformin on tumor microenvironment, Seminars in Cell and Developmental Biology 98 (2020) 90–97
25. Liu, A., et al, Correlated Alterations in Prostate Basal Cell Lauer and Basement Membrane, Int Jrl Bio Sci, V 5, 2009, pp 276-285.
26. Liu, Q., et al, Metformin Inhibits Prostate Cancer Progression by Targeting Tumor-Associated Inflammatory Infiltration, Clin Cancer Res; 24(22) November 15, 2018
27. LoPiccolo, J., et al, Targeting the PI3K/Akt/mTOR Pathway, Drug Res Up, 2008, pp 32-50.
28. Loubiere et al, Metformin-induced energy deficiency leads to the inhibition of lipogenesis in prostate cancer cells, Oncotarget, Vol. 6, No. 17, March 10, 2015
29. Margel, D., et al, Metformin Use and All-Cause and Prostate Cancer Specific Mortality Among Men With Diabetes, JCO Sep 1, 2013:3069-3075; DOI:10.1200/JCO.2012.46.7043.
30. Marks, F., et al, Cellular Signal Processing, 2nd Ed, Garland (New York), 2017
31. Mendelsohn, et al, The Molecular Basis of Cancer, Elsevier (New York) 2014.
32. Mihaylova, Maria, Reuben J. Shaw, The AMPK signalling pathway coordinates cell growth, autophagy and metabolism, Nature Cell Biology13, 1016–1023 (2011) doi:10.1038/ncb2329
33. Saad et al, Treatment of nonmetastatic castration-resistant prostate cancer: focus on second-generation androgen receptor inhibitors, Prostate Cancer and Prostatic Diseases, 8 Feb 2021
34. Saber et al, Exosomes are the Driving Force in Preparing the Soil for the Metastatic Seeds: Lessons from the Prostate Cancer, Cells, 28 Feb 2020
35. Saxton and Sabatini, mTOR Signaling in Growth, Metabolism, and Disease, Cell, March 9, 2017
36. Shackelford, D., Reuben J. Shaw, The LKB1–AMPK pathway: metabolism and growth control in tumour suppression, Nature Reviews Cancer 9, 563-575 (August 2009) | doi:10.1038/nrc2676
37. Shao, et al, Inhibition of Polo-like Kinase 1 (Plk1) Enhances the Antineoplastic Activity of Metformin in Prostate Cancer, J. Biol. Chem. 2015 290: 2024-2033.

38. Shaw, R., The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress, PNAS March 9, 2004 vol. 101 no. 10 3329–3335

39. Shen and Rubin, Prostate Cancer, CSHL Press, 2019

40. Talty and Olino, Metabolism of Innate Immune Cells in Cancer, Cancers 2021, 13, 904

41. Wang et al, Metformin suppressed tumor necrosis factor-α-induced epithelial-mesenchymal transition in prostate cancer by inactivating the NF-κB signaling pathway, Transl Cancer Res 2020

42. Weinberg, The Biology of Cancer, 2nd Ed, Garland, 2014

43. Whitburn et al, Metformin and Prostate Cancer: A New Role for an Old Drug, Curr Urol Rep (2017) 18: 46

44. Xie et al, Metformin and Androgen Receptor-Axis-Targeted (ARAT) Agents Induce Two PARP-1-Dependent Cell Death Pathways in Androgen-Sensitive Human Prostate Cancer Cells, Cancers, 2021, 13, 633

45. Zamagni et al, Non-nuclear AR Signaling in Prostate Cancer. Frontiers in Chemistry, 26 Feb 2019

46. Zhao et al, Therapeutic Repurposing of Biguanides in Cancer, Trends in Cancer, 2021

47. Zheng, L. et al, NF-kB Regulates Androgen Receptor Expression and Prostate Cancer Growth, Am Soc Invest Path, V 175, 2009, pp 489-499.

48. Zingales et al, Metformin: A Bridge between Diabetes and Prostate Cancer, Frontiers in Oncology, October 2017

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