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Review

Is the Response of Tumours Dependent on Dietary Input of Some Amino Acids or on the Ratios Among Essential and Non-Essential Amino Acids? All That Glisters is Not Gold

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Abstract: Production of energy is a main task of cancer cells metabolism, since costs of duplicating are enormous. Although energy is derived in cells by dismantling carbon to carbon bonds of any macronutrient, cancer nutritional needs for energetic purposes have been studied primarily as dependent on glycolysis. Since the end of the last century, awareness of dependence of cancer metabolism on amino acids not only for protein syntheses but also for matching energy needs has grown. The roles of specific amino acids, like glutamine, glycine and serine have been explored in different experimental conditions and reviewed. Moreover, there are epidemiological evidences that some amino acids used as supplement for therapeutic reasons (the branched chain ones) may reduce incidence of liver cancer, and some molecular mechanism has been proposed as functional to their protective action. On the contrary, metabolic signature of some pathology connected with increased risk of cancer, like prolonged hyperinsulinemia in insulin resistant patients, is signed by plasma elevated levels of the same branched chain amino acids, posing puzzling questions to clinicians. Most recently, peculiar formulations of amino acids, deeply different if compared to amino acids compositions normally present in foods, have shown the power to master epigenetics slowing growth or driving cancer cells to apoptotic death, while being even beneficial for normal cells and for animals health and life span. In this review, we will analyze and try to disentangle some of the many knots dealing with complexities of amino acids biology and linked to cancer metabolism.

Keywords: amino acids; cancer; energy metabolism; autophagy; apoptosis; glutamine; diabetes type 2

1. Introduction

Since Otto Warburg described the abundant amounts of energy necessary for duplicating, and derived in tumours from cytoplasmic glycolysis to lactate, even in aerobic conditions [1], the finalistic advantages of incomplete glycolysis for cancer cells and reduced mitochondrial glycolysis, the so called "Warburg effect", are still debated [2,3]. Cancer cells main task is to duplicate, and this requires both energy and substrates adequate for completing synthesis of any structure allowing formation of new cells. Therefore the quality and quantity of substrates present in the environment either drives fate of normal cells [4], and probably selects cancer cells since their beginning [5]. Protein syntheses are the most energy demanding since any peptide bond has a cost of 4 ATP [6], and among the few advancement in understanding the peculiarities of energy production by cancer metabolism, most are related to the observation that cancer cells use glutamine far beyond the need for protein syntheses [7].

Some of the controlling and controlled paths matching energy and substrates for syntheses have been identified, and different kinases studied in both normal and cancer cells[8,9,10]. Attempts to find molecules ruling metabolic pathways at key points, so that if blocked they would impair selectively cancer cells development and survival, is restless, but most failed [11] since till now few molecules proved to be efficient on inhibiting cancer development, and those are significantly toxic for normal cells [12,13,14,15].

In the new century, attention to amino acids metabolism and protein synthesis in cancer has grown, and most researchers focused on the possible role on cancer cells growth of different non-essential (NE) amino acids (AA), since their carbon skeleton may provide key intermediates to energy metabolism. Accordingly, renewed attention to some specific NEAA metabolic pathway has led to discover biochemical interactions not described previously, such as the allosteric activation of pyruvate kinase [16] (the enzyme that de-phosphorylate to pyruvate the terminal glycolytic intermediate Phospho-enol-Pyruvate) by exogenous serine, and whose activation and significance in cancer is still controversial[17].

Although historic studies identified the source of amino acids used by cancer in the same tissues, which worked as a reservoir during prolonged starvation [18], amino acids peculiarly important for cancer energy metabolism and syntheses are considered: proline, glutamine, glycine and serine, the latter three most abundant in food proteins . About glycine and serine, that for instance are metabolically strictly related, a very recent report suggests that eliminating them by diets would antagonize tumour development, based on data both in vitro and in vivo [19].

On the contrary, few weeks later, another study reported that two formulations composed mainly of EAA , and peculiarly one providing also serine and ornithine-αketoglutarate (αKG), were similarly efficient in inducing apoptosis in different cancer cell lines. This study showed that EAA in excess of NEAA creates a paraphysiological condition of substrates availability, unlikely available in natural environments, which is healthy for normal cells but unveils a peculiar fragility of cancer cell [20], which evidently evolves selecting clones [21] adapted to environmental nutritional conditions where NEAA are always prevalent, and so cancer cells are fully dependent on those for surviving and multiplying. The study was planned to explore the hypothesis that a most relevant competitive advantage of cancer cells is based on prevalent availability of NEAA, far most abundant in food and body proteins than the EAA necessary for syntheses, thus reducing energetic costs, since producing NEAA would consume energy and subtract key glycolytic intermediates necessary to provide carbon backbone to newly synthesized NEAA . This behavior would rule the activation or inactivation of processes connected with survival like autophagy or proteolysis , which are used by cancer at the purpose of maintaining energy and substrates necessary for rapid duplication. A balance which is controlled by the usually far less EAA availability. Inversion of the EAA/NEAA ratios unveils the dependence of cancer cells on environmental abundance of NEAA, since when EAA are sufficiently present to drive syntheses, proteasome-dependent proteolysis is inhibited, but autophagy is triggered to match NEAA needs adequately to maintain syntheses at the pace dictated by EAA availability, outdoing the concept of cross-linked and compelling reciprocal controls predicted by actual knowledge on mTOR and AMPK relationship and inhibition or activation of autophagy. The proteasome inhibition exerted by EAA, different from that exerted by drugs like bortezumib or carfilzomib [22], is peculiarly evident and limited to cancer cells. Furthermore EAA have already been shown to be protective against doxorubicin heart damages [23], characterized by disregulation of mitochondria and autophagy [24], and being also efficient in ameliorating heart performance in aged diabetic patients affected by heart failure [25]. Introduction of serine and ornithine- αKG, in specific ratios with EAA, seems efficient to further implement the effects of altering environmental EAA/NEAA, and this not so predictable result is peculiarly efficient in eliciting activity and control of the endoplasmic reticulum proteases, and specifically by inducing cathepsin-L, mainly involved in triggering autophagy.

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Is it possible that two different experimental protocols produced so similar results and so different conclusions by the authors? Which is the role of serine and glycine and why have been so frequently involved in cancer metabolism? Which is the biochemical relationship among serine-glycine pathway, glutamine and proline and energy metabolism?

2. Nutrition and the Risk of Cancer: The Puzzling Question of Insulin Resistance and Diabetes Type 2. Amino Acids Plasma Patterns Are Cause or Effect?

The matter is relevant since insulin resistance and hyperinsulinemia, unfortunately intertwined with diabetes type 2, are considered risk factors for cancer, and this risk has been linked to insulin hyper-activation of PI3K/AKT/mTORC1 signaling [26]. Thus, in a recent report Fontana and al. underline the possible role of increased insulin resistance detected when BCAA were supplemented in the context of a high-fat diet in rats [27], although they do not further discuss the finding that BCAA have no effect on insulin resistance when summed to normal chow observed in the same paper they cited [28]. As well as it is ignored another report showing that by doubling dietary leucine intake was significantly reduced either high fat diet induced weight gain, and were also improved hyperglycemia and hypercholesterolemia since also insulin resistance was reduced [29]. We may also observe that in humans, one double blind controlled study on long term therapy with BCAA supplementation in diabetic patients, that found positive results on glucose tolerance and on a typical feature of insulin resistance, liver steatosis [30] , and also another study showing that acute ingestion of BCAA elicits efficient insulin response and consequently hypoglycemia [31], were also not considered and discussed. To further clarify the item, a very well designed study showed that leucine supplementation in high fat diets would have different effects on glucose tolerance and insulin resistance if studied on short or long term, when beneficial effects are unequivocally evident. In the first 24 weeks, mitochondria damages by fat loads prevail and overwhelm positive effects of leucine supplementation observed only after 32 weeks, when mitochondria biogenesis and glucose tolerance are restored by leucine [32].

Still, , worth of notice, there are contrasting opinions on the role of essential amino acids in promoting health and lifespan [33]. Although it was proved in animal settings that supplementation of EAA , rich in BCAA increases life span by protecting mitochondrial biogenesis and implementing mitochondria efficiency-related systems (i.e.: eNOs, cytochrome c, ROS and antioxidant defenses expression) [34,35] , Fontana et al. claim that branched chain amino acids restriction is a possible promoter of health, an hypothesis based also on the preliminary observation that plasma profiles of diabetes type 2 patients are characterized by high levels of BCAA [27].

An elevation of branched chain amino acids in plasma of type 2 diabetes patients is often suspected to have causal role in diabetes by a part of researchers, and so elevated BCAA in plasma are not considered , on the contrary, as prevalently a potential biomarker of altered metabolism following reduced peripheral catabolism of BCAA. This acritical interpretation of metabolomics is puzzling for the experts in amino acids metabolism, since in type 2 diabetes patients both BCAA transaminase and branched chain ketoacids de-hydrogenase are decreased in adipose tissue, as well as is diminished mitochondrial oxidation in peripheral muscles [36], and the sum of those modifications explain plasma BCAA elevation in those patients.

Of notice, in another pathological condition also characterized by presence of insulin resistance and altered glucose metabolism, liver cirrhosis, BCAA in plasma are so often depressed that they are related to a specific metabolic alteration of brain function, a syndrome known as hepatic encephalopathy [37]. Actually, still there is no clear explanation for the diverging plasma patterns of BCAA in those two clinical pictures characterized by insulin resistance, but we obviously may link the differences to metabolic insufficiency and altered haemodynamics of a damaged liver.

As well, it is not of help in clarifying the picture the extremely complex protocol of Fontana et al. [27]. They reduced to 7% nitrogen content of diets in mice and compared the consequent physical and biochemical modifications, with findings observed in a control group fed with 21% grams of

amino acids. Calculated from their tables, amino acids provided by diets were 7,75 grams and 24,45 grams respectively . In percentage of total nitrogen, diets provided 56-62% of NEAA, according if cysteine and tyrosine are considered or not functionally as NEAA or as EAA . Consequently BCAA representing 39 - 44% of all EAA, always depending on how cysteine and tyrosine would be classified functionally. Indeed, both those amino acids may be considered somehow essential in cell cultures, since tyrosine is indispensable for all cells but for liver and partially kidneys, the only organs containing the hydroxylase of phenyl-alanine providing tyrosine synthesis, while sulphur containing amino acids are most safely provided by balanced methionine and cysteine-cystine stoichiometric ratios, so protecting folates metabolism and minimizing homocysteine syntheses due to methionine metabolism to match cysteine requirements [20] . Following a reduction of nitrogen intake to one third, in front of a near 30% increased daily caloric intake per gram of body weight when deprived diets were given, they observed a continuous drop in body weight by losses of both fat and lean body mass. Those findings could identify an efficient model of "sarcopenia [38] leading to wasting [39]" protocol, but this achievement was not discussed. On the contrary, although animals fed control diets had normal glucose tolerance, authors identified an "improved glucose tolerance" related to a lowered glycemia and insulinemia in animals fed the near 7% protein diets and with losses of muscles and adipose tissue, when compared to controls. Any effect on glucose, insulin and on muscle and fat wasting were abolishedand findings were comparable to controls, by a further diet containing 21% amino acids, the same amount provided in control diets, but the formula was modified and contained enormously increased amounts of some neoglucogenic non-essential (NE) AA (glycine near 5 times, proline and serine 2,7 times, alanine more than 2 times, and aspartic acid was increased near of 30%) while providing markedly (30% or less) reduced amounts of other NEAA (arginine, cysteine, glutamine, tyrosine) and of all essential amino acids, with the exception of BCAA, whose content was not modified. Thus, in this last diet, BCAA were contained in the same amounts provided by control diets, and so in extremely elevated ratios to all other essential amino acids . To the skilled biochemist is sufficient to remember both the allosteric inhibition exerted by alanine on both 6-phosphofructo-kinase and pyruvate-kinase, and also the competitive relation of alanine with pyruvate on pyruvate transporters for mitochondrial entry, for understanding why, although arginine was reduced, the overwhelmingly increased amounts of neoglucogenic glycine, serine, aspartic and glutamic acid would have blunted any other biological effects of EAA, and any eventually observed alteration would not be purely attributable to the elevated amounts of BCAA provided by the peculiar diet [27]. But hyperinsulinemia and hyperglycemia were not detectable when compared to control diets, and the hypothesis of a protective effect of BCAA against those kinds of dietary amino acids manipulation has not even taken into account by the authors.

Although there are evidences proving that alimentary amino acids - that is the sum of EAA and NEAA in the ratios contained in foods- may promote insulin resistance [40], an argument discussed in details elsewhere [41], they showed by this complicate protocol that a marked increase in BCAA even in those demanding conditions would not be associated with rising insulinemia and glycemia at levels different from what observed by control diets. Still, they claimed even by the title of their paper that decreased consumption of BCAA improves metabolic health.

Since we are conducting by a very straight and rigorous protocol life span studies dealing with varying EAA/NEAA ratios at 15% steps in diets providing from 100% NEAA up to 100% EAA, it would have been interesting to have data on length of lifespan of animals wasted by the 7% protein diet, and if, at necroscopy, alterations of both weights and hystochemistry of internal organs have been detected, and if those matched with what they defined "improved glycemic control" when compared to insulinemia and glycemia registered in normal animals fed normal diets. Indeed, should it be considered an improvement, that can be linked to increased life span, the reduction of glycemia and insulinemia in healthy animals, following diets providing a marked nitrogen restriction and signed by caloric increase, but so deprived of nitrogen to induce both loss of adipose tissue and muscles mass? The question posed by Fontana et al. deals with the definition of normality, and is extraordinarily relevant, since they suggested a finalistic interpretation of changes of glycemia and insulinemia found in wasted animals that have to be compared to those we would

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usually have considered normal, since obtained in normal animals with normally active beta cells, fed with normal diets, and not in animals affected by insulin resistance.

It would be interesting to answer this question, implications are enormous for defining and understanding the relationship among nitrogen intake, body composition and lifespan. It would be important also to clarify metabolic systems controlling syntheses either of fats in adipose tissue and of proteins in muscles, since peculiar modulations of signaling paths linked to insulin by different quality of nitrogen intake may have also implications on cancer risks and development [42].

3. Essential Amino Acids and Cancer: What if Environment Changes the Rules?

All mammals develops from a single cell, one oocyte fecundated by one spermatozoon. All cells in all tissues and organs have origin from rapid duplication of that cell, and all cells have to withstand to the same rules that allowed development during fetal life: matter and energy should be constantly available in adequate amounts to promote survival of any cell, and so maintaining integrity of the organism. We have incomplete understanding about both how memory and mechanisms of this primordial time of our lives survive into cells, and how those are controlled by specific regulatory proteins peculiarly in dividing cells [43]. Although protein synthesis suitable to match duplication needs is taken for granted, very few studies dealing with dependence of proliferation of cancer on essential amino acids have been published, and among the few, quite only the role of specific amino acids have been studied.

As an example, recently, and so peculiarly worth of notice, is a report on metabolic dependence of embryonic stem cells on threonine, indispensable for biosyntheses but also for controlling signaling paths that allows pluripotency and self-renewal. Embryonic stem cells have many features similar to cancer cells: for growth and proliferation both require catabolic transformation of nutrients into energy but contemporarily metabolic building blocks to meet the biosynthetic and anabolic needs. Also embryonic stem cells use glycolysis to lactate as the preferred energy metabolic route to generate ATP , the metabolic adaptation known as “Warburg effect”. Accordingly, embryonic stem cells reportedly have less developed mitochondria and lower oxygen consumption. Therefore, Chen and Wang [44] described the possible role in rapid dividing cells of threonine-dehydrogenase mediated catabolism of threonine to glycine and acetylCoA, with the latter entering citric acid cycle, and promoting fatty acid syntheses through conversion in malonylCoA into mitochondria and export in cytoplasm. In turn, glycine would contribute to one carbon metabolism and methylations paths, fueling purine syntheses [45], as would be discussed in details for serine/glycine metabolism in a specific paragraph.

Also some aspects of catabolism of tryptophan have been related specifically to cancer, and so depletion by indoleamine-2,3-dioxygenase (IDO) expressed either in tumor cells or antigen-presenting cells, and also production of an end product like kinurenine has been related to immuno-suppression in cancer microenvironment and in lymph nodes draining it, so inducing T-cells anergy and apoptosis facilitating cancer expansion [46].

On the contrary, follow up studies of large populations have discovered that obese cirrhotic patients treated with high doses of branched chain (BC) amino acids (AA), at the purpose of preventing hepatic encephalopathy, had a reduced risk of liver cancer [47] . As possible mechanisms for those results was suggested that BCAA may act by inhibiting insulin-induced PI3K/Akt signaling pathway through active regulation of the serine/threonine kinase involved in control of protein syntheses, cell growth and metabolism, named mammalian target of rapamycin (mTOR) , targeting its functional complex mTORC1, which activates a feed-back loop on PI3K signaling, and inhibiting the other complext mTORC2 , and so the controlled genes related to apoptotic and anti-apoptotic pathways. The sum of those effects, finally, would foster cancer cells apoptosis [48].

Those observations are of interest since different essential amino acids, leucine first of all, but also isoleucine, methionine and less potently valine, control on mTORC1 activation of protein syntheses is widely accepted [49].

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4. Reducing EAA/NEAA Ratios Drive Cancer Cells to Apoptosis Activating Autophagy and Inhibiting Proteasome.

Indeed, we have shown that different types of cancer cells do not survive in an environment where EAA are largely prevalent on NEAA [20], an environment clearly beneficial for normal cells used as controls. A clear summary of amino acids contained in the two formulations used in those experiments are reported in Table 1. Since some cancer cell has been shown to lose enzymes for synthesizing some specific non essential amino acid (such as arginine, by cells lacking arginino-succinate synthase (, AS-Sinth), indispensable for recycling citrulline to arginine) [50] while have to implement synthesis of some NEAA, even if abundant in food and in circulating fluids, as serine/glycine, the hypothesis was that cancer spares on NEAA syntheses, since it develops adapted to what present most abundantly, in excess of EAA, in the environment. We suspected that cancer cells would not be suitable to manage specific alterations of EAA/NEAA ratios, as opposed to normal cells which maintain an expensive machinery to adapt to shortages of any NEAA and to sudden availability of most scarcely available EAA. Indeed we survive with limitations to our well being even if undergoing caloric restriction, but we respond rapidly to EAA availability with increased syntheses even in chronic pathologies dominated by wasting [25]. Therefore, we showed that significantly reducing the EAA/NEAA ratio in the environment, inhibits in cancer cells the proteasome linked proteolysis, while contemporarily increasing autophagy, triggering an apoptotic drive [20] ,a response to peculiar stimuli that has already been analytically described, and seems beclin-1 dependent [51].

Table 1. AAs ratios in the formulations tested by Bonfili et al. (FEBS J, 2017, doi: 10.1111/febs.14081, citation 23), expressed as percentages of 100 grams.

100% EAAs (w/w %):

Leucine	Isoleucine	Valine	Histidine	Lysine	Threonine	Methionine*	PhenylAlanine
31.25	15.625	15.625	3.75	16.25	8.75	1.25	2.5
Triptophan	Tyrosine**	Cystine*					
0.5	0.75	3.75					

85% EAAs and 15% NEAAs (w/w %):

Leucine	Isoleucine	Valine	Histidine	Lysine	Threonine	Methionine*	PhenylAlanine
13.53	9.65	9.65	11.60	11.60	8.70	4.35	7.73
Triptophan	Tyrosine**	Cystine*	Serine	NAcetylCysteine	Ornithine-αKetoGlutarate		
3.38	5.80	8.20	2.42	0.97	2.42		

Table 1. Detailed description of amino acids compositions, providing mostly essential amino acids (EAA) and supplemented to HCT116 cancer cells. Bonfili et al. and effective in inhibiting proteasome while triggering autophagy dependent apoptosis [20].

*Both formulations contain cystine (and in 85% NEAA and 15% EAA formulation also N-acetylated cysteine) summed to methionine to match sulfur-containing AAs needs minimizing possible homo-cyst(e)ine toxicity.

**Tyrosine is present in both formulations, since, when calculating phenylalanine needs, it was considered that tyrosine is a NEAA only for the liver and partially for kidneys, which can derive it by hydroxylation of phenylalanine, whereas it is fully essential in any other cell of the body, including HCT 116 colon cancer cells.

Implications of these data are noticeable: for the first time it is described a peculiar fragility of cancer cells to modifications of environment that are on the contrary favorable for normal cells, as documented in animals and humans and shown by previous findings [52,53,54] , since EAA/NEAA

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triggers in a variety of cancer cells poly-adenosyl-di-phosphate ribose polymerase-1 (PARP-1) cleavage paralleled by activation of caspase 3 and drive them to apoptosis. Also, increased EAA/NEAA ratio finalistically modulates cathepsin B and L activity, and also increases p53, p62SQSTM1 and, as said, beclin 1 and LC3BII which witnessed autophagy activation. Also, EAA supplementation generated a marked inhibition of proteasome activity. Since EAA formulations proved efficient in chronic heart failure clinical settings [55], while actual drugs promoting proteasome inhibition proved to be extremely toxic on cardiac cells, our findings open a new avenue of possibilities in supporting or enhancing chemotherapy effects, while protecting target organs, like heart, from jatrogenic damages [56,57].

5. Serine and Glycine: Guilty of Feeding Cancer or Innocent Intermediates of Metabolism ?

Either one carbon metabolism is necessary for nucleotides and NADPH production, and also methyl-groups transfer from donors is required to target modifications of DNA, RNA and proteins, all depend on metabolism of serine. Serine is an interesting amino acids, commonly present in food proteins, but it is a gluconeogenic amino acid easily synthesized, since its carbon skeleton is derived by 3-phospho-pyruvate originated by anaerobic glycolysis from glucose, then transaminated on alpha carbon by a glutamine-3 phospho-pyruvate transaminase (PSAT1) and finally dephosphorylated by a phospho-serine dephosphorylase (PSPH) to serine. Some cancer depend on enhanced activity of this synthetic pathway to maintain purine syntheses and, using specific inhibitors of endogenous serine synthesis, cell growth is retarded even in presence of abundant exogenous serine [58]. Serine can also be derived by glycine, but this reaction consumes NADH and depletes of methyl group folates in a metabolic path providing modifications similar to those consequent to alcohol ingestion, a condition indeed also marked by homo-cysteine accumulation [59]. That ethanol consumption is linked to increased risk of cancer is well known [60], but the role of methyl groups depletion of folates is more puzzling. Indeed, on one hand folate fortification of food has reduced significantly the incidence of colon cancer in US on long term epidemiological survey [61], thus, repletion of folate reserves is favorable to preventing some cancer, on the other hand, very high level of folates intake does not further improve protection [62] . For sure the anti-folate molecules, developed on the clinical observations that dietary folate deficiency reduced leukemic cell number in acute leukemia [63] are still one of the most efficient and widely used drug in chemotherapy of different tumours [64]. Therefore, since maintenance of folates efficiency is based on continuous reloading of methyl-groups in position 5 and 10, and those methyl groups are necessarily derived by serine to glycine metabolism through an energetically favorable reaction since produces NADH, peculiar roles of serine in cancer has been repeatedly suggested [65,66]. Transfer of methyl groups is fundamental for metabolism integrity, and some congenital alterations of the folate cycle expose to increased cancer risk [67]. Although the different positions of methyls on tetra-hydro-folate molecule signs their destinations, the dependency on serine for fully reloading and reactivating folates efficiency is valid for both methyls, either in 5- or 10- position on tetrahydro-folates (THF) molecules . Methylation is required for nucleotide synthesis, which is increased in cancer since related to rapid duplication, thus much work has focused on serine/glycine role in cancer cells metabolism on the hypothesis that by depriving of serine/glycine cancer metabolism, growth and development of cancer would be impaired. Since depletion of methyls from THF is rapidly obtained by ethanol ingestion, and this is connected with increased cancer risks in different tissues [68], there is some contradiction in how we look at what happens in cancer. Indeed, often researchers blame utilization of single pathways indispensable for normal metabolism which are implemented although not structurally modified to fulfill cancer cells specific metabolic needs. From adenosine triphosphate (ATP) to membrane lipids syntheses, many if not all normal metabolic paths have to match an increased duplication need marking cancer cells aggression. In our opinion it should not be forgotten that cancer cells have origin from normal cells of normal tissues of normal organs, and developed substantially bound to the same metabolic rules and activities of cells they

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derived from, but, evidently, with some metabolic advantage not yet evidenced that competitively select them [17]. There is a peculiar history of papers focusing on serine/glycine deprivation [19, 69] and showing that cancer cells suffer metabolic derangement and lowered development when fed formulation deprived of serine and glycine. But, based on data presented also in an interesting publication [27], the serine/glycine deprivation protocol is heavily biased by methodology, and authors have fallen into a Stolzemberg's trap [70] since they compared a formulation containing either EAA and NEAA, comprising serine and glycine among the NEAA, to the same formulation simply deprived of serine and glycine. Doing so, they made a methodological mistake since they did not maintain constant EAA/NEAA ratios by substituting serine and glycine with some other NEAA of their choice. Thus, they have compared two metabolic substrates fully different and not principally for the serine and glycine deprivation. Indeed, to appropriately test the hypothesis that serine and glycine are responsible for maintenance of cancer cells and to test a formulation deprived by serine and glycine correctly, differently by the protocol they used, they should have deprived the control formula of serine and glycine, but those amino acids should have been substituted with equimolar amounts of, as an example, glutamine, aspartate, glutamic acid or at worst arginine for serine, and alanine for glycine. On the contrary, they tested not simply a formulation deprived of serine and glycine, but a formulation providing overwhelming EAA content respect to NEAA, thus an altered EAA/NEAA ratio, and still attributed results to the simple substitution of serine and glycine, as resumed in figure 1.

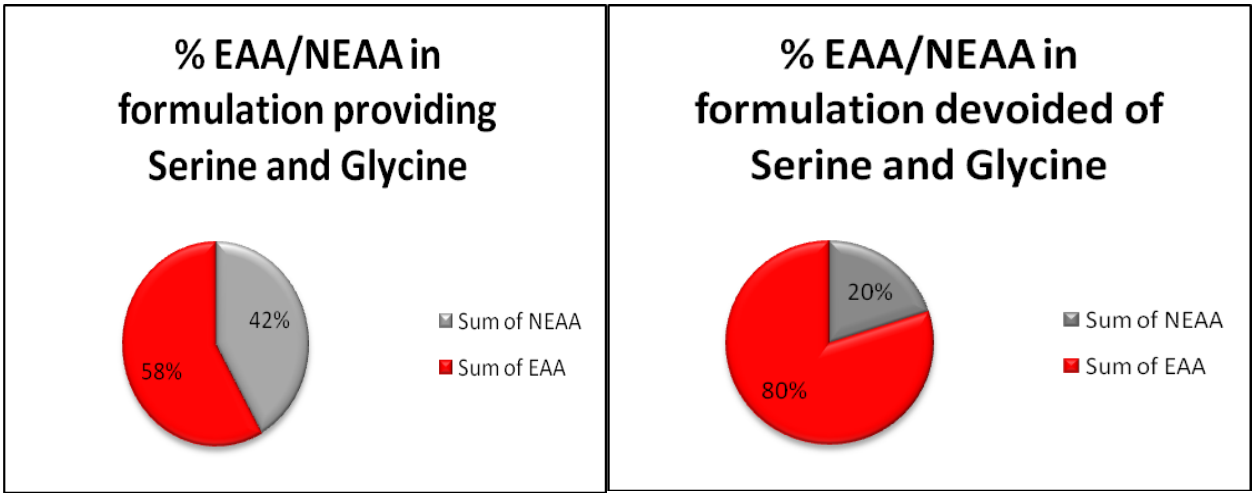

Figure 1. Figures are based on methods used according Maddocks and al., citations 22 and 39. Composition o f "Amino acid pre-mix", providing both serine and glycine : Arginine-HCl: 1,60%, L-cystine: 0,64%, L-Glutamine:1,60%, glycine: 1,33%, L-histidine-HCl: 0,80%, L-isoleucine: 1,07%, L-leucine: 1,60%, L-lysine-HCl : 1,87%, L-methionine:0,80%, L-phenylalanine: 1,07%, L-serine: 1,33%, L-threonine: 1,07%, L-tryptophan: 0,27%, L-tyrosine: 0,53%, L-valine:1.07% . Composition of "Amino acid premix" devoided of serine and glycine: L-arginine:-HCl: 1,60%, L-Cystine: 0,64%, L-glutamine: 1,60%, L-histidine: 0,96%, L-isoleucine: 1,28%, L-leucine: 1,92%, L-lysine-HCl: 2,24%, L-methionine: 0,96%, L-phenylalanine: 1,28%, L-threonine: 1,28%, L-tryptophan: 0,32%, L-tyrosine: 0,64%, L-valine: 1,28%. Weight ratios among EAA (in red) versus NEAA (grey) provided by the two formulations as derived by Methods, in Maddocks OD et al. Serine starvation induces stress and p53-dependent metabolic remodeling in cancer cells. Nature.2013. 493, 542-546. It should be noticed that percentages have been calculated on exactly 16 grams of the formulation Serine and Glycine free, and on 16,28 grams which is the sum of weights of the amino acids declared in the formulation providing both Serine and Glycine. Ovalbumin, the reference protein for human nutrition, contains 44.4% of EAA, or EAA/NEAA ratio is approximately is 0.8. On the contrary, in formulations used by Maddocks and al. (according citations 22 and 39), if content of amino acids would have been calculated in molar ratios (number of molecules), the EAA /NEAA molecular ratio would have been near 1.2 when serine and glycine were provided, and near 2.9 when the formulation was modified

by eliminating Serine and Glycine. In any case essential amino acids content of those formulations is quite far from food content of amino acids.

Since we described [20] some of the mechanisms through which increased EAA/NEAA ratios alters the strict environment in which cancer cells metabolically predominates, by altering EAA/NEAA ratio was unveiled a fragility of cancer metabolism that we identified in the dependence on largest availability of NEAA respect to EAA. Those findings were observed by inverting the EAA/NEAA ratio usually present in physiological conditions (as in plasma and extracellular fluids) and reflected both in human cells and in foods proteins of any origin. Therefore, by supplementing two different formulations in which EAA were largely prevalent, we evidenced a significant activation of autophagy. This is probably linked to a reduced capacity of cancer cells in deriving sufficient NEAA , from intermediates of glycolysis,to match the huge needs for duplication. Increased EAA in cancer cells environment would imbalance nuclear signaling controls, as we showed at least at the level of poly ADP-ribose polymerase-1 (PARP-1), and this trigger a cascade of events mortal for cancer cells, since metabolic consumption of NEAA in cancer is devoted to match both energy production and syntheses of proteins, and this, as a consequence, triggers autophagy. EAA in excess also blunt proteasome-dependent proteolysis, and this critical condition conflicting with increased autophagy finally triggers apoptosis. The substantial alteration of EAA/NEAA ratio used in our study [20] is similar, even if most marked than that reproduced without full conscience, to that used by Maddocks and al. [19].Therefore, since alteration of NEAA/EAA ratios was also induced by their protocol, their most interesting results were misleadingly attributed only to deprivation of serine and glycine. The methodological mistake was that deprivation of serine and glycine from the amino acids formulation used in control groups was done without compensating weight or molar ratios with other NEAA, so they compared totally unbalanced EAA/NEAA ratios. A schematic representation of the EAA and NEAA contents in formulations used according to Maddocks and al. , is presented in Figure.1.

It is interesting to notice that presence of some serine, as well as of some ornithine (precursor or product of glutamine metabolism), linked to alpha-ketoglutarate (OKG), the physiological precursor of glutamic acid and glutamine synthesis, potentiates the effects of the EAA rich formulation and elicits specific epigenetic responses , one example is the peculiar and different cathepsin B and L activation . EAA rich formulations supplemented to diet have already been shown to increase life span by counteracting age-linked mitochondrial biogenesis losses [34], and different papers have shown, both in experimental and in human settings, that by lowering EAA/NEAA ratios with some essential amino acids (the branched chain ones) , liver damages and cancer risks are reduced [32,71]. Details of the mechanisms underlying those behaviors are entangled and still under study [43], although we think that by altering EAA/NEAA ratios a mean of triggering a totally innovative network of relationships among key gatekeepers as mTORC1 and 2 [72], AMPK and AKT [73] , has been identified, unveiling some control of either proteolysis/autophagy network mastered by amino acids availability , and is inrelationship with apoptosis (and/or anoikis ? [74]).

6. Glutamine, Proline and Ornithine, an Entangled Relationship-

If serine is a key amino acid for maintenance of methylations, that is by one- carbon linked biochemistry, glutamine is pivotal due to the relevance of its role as nitrogen donor. Thus glutamine is central for synthetic pathways driving to NEAA syntheses, albeit its carbon skeleton is extremely important also in supplying intermediates to citric acid cycle, peculiarly providing α-ketoglutarate to citric acid cycle and so refueling of a key oxaloacetate precursor the mitochondrial acetate oxydation.

Three different and excellent reviews have been published quite contemporarily on the role of glutamine metabolism in cancer development and survival. All reviews underscored how glutamine is necessary for purine and pyrimidine biosynthesis, beyond being necessary for completing protein and also glucosamine syntheses in extra-cellular matrix, required by cancer cells growth and

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duplication [7, [75] , [76]]. Glutamine is an amino acids synthesized easily in all cells containing transaminases. It is present also in erythrocytes, since from the intermediate of glycolysis α-ketoglutarate (αKG) first glutamate is synthesized by transamination of one –NH_2 group on carbon α and obtained dismantling nitrogen from aspartate or alanine, but also from an essential amino acids like leucine or valine.Then, glutamine is completed by glutamino-synthase which transfers another amino group on carbon γ of glutamic acid. As already said, in turn, glutamine has a central role in syntheses of all NEAA since providing nitrogen for amination of different substrates coming from glycolytic metabolism, like pyruvate, oxaloacetate and αKG. The pathway works in both ways, thus from glutamine different key molecules connected with refueling energy production, such as pyruvate, oxaloacetate or αketo-glutarate, can be generated by transaminases that transfer –NH_2 thus providing maintenance of NEAA synthesis when and where necessary, or by transamidation that generates NH_3/NH_4.This metabolic path is peculiarly important in kidneys were excretion of NH_4 allows control of K and Na reabsorption [[77]]. Glutamine, like the metabolically related amino acid arginine [[78]], in conditions of elevated metabolic requirements may become "conditionally essential" since largely consumed for producing energy and thus may become insufficiently available for maintenance either of immune defense (lymphocytes and macrophages proliferation and metabolism) and absorption of nutrients to prevent malnutrition (enterocytes). Glutamine is also indispensable due to its role both as energy substrate and as precursor of nucleotides syntheses, thus necessary in maintenance of physiological rapid duplication for cells like enterocytes, but also for leucocytes in life threatening conditions, as in sepsis [[79]]. On the other hand, it has been clinically proven that EAA supplementation is highly effective in promoting immune system efficiency in those same conditions [[80]], and this may be an alternative to glutamine supplementation since EAA can promote glutamine endogenous synthesis locally, but also protects against ammonia and glutamine dependent brain damages following excess ammonia production by enterocytes, on the contrary of what happens with enterally provided glutamine [[81]]. Indeed, in normal conditions enterocytes prevent glutamine absorption dismantling by deamination of glutamine molecules by a specific phosphate-activated glutaminase, prior to absorption of glutamate and so producing ammonia which can be absorbed and generate most severe neurological alterations in case of liver impairment [[82]]. This chain of events unveiled the toxicity of ammonia-dependent glutamine synthetic rate in neuronal/astrocytes metabolism and the protective role of some EAA, peculiarly leucine that activates export of glutamine out from brain cells so treating hepatic encephalopathy [[83]]. Accordingly, the adverse effects of long term glutamine supplementation have been largely discussed [[84]], in brief they may be summarized with observing that exogenous glutamine suppresses expression of glutamine-synthase [[85]], so patients treated with glutamine supplementation should be discontinued by carefully queuing therapy, or a sudden stop in supplementation would have as consequence of impaired endogenous synthesis either glutamine starvation and loss of immune efficiency [[86]].

In cancer cells, glutamine is consumed far more than predicted by demands referable to protein synthesis needs [7], thus increased requirements dealing with energy costs may be suspected, and although various attempts to target glutamine metabolism on cancer-specific glutaminase isophorm and its variants have been explored , the understanding of the full picture of requirements of glutamine in tumour cells appears far from being completed. Also, metabolic heterogeneities linked to genetic instability and both progression of mutations and the complexity of tumour micro-environment interface indicates the need for more studies to understand regulation of tumour metabolism and if the complex biochemical network where glutamine is at the center of anaplerotic and cataplerotic metabolism would be a suitable target for effective therapies [79].

Also, glutamine metabolism is extremely intertwined with synthesis of many other different amino acids, such as ornithine and proline, whose origin is directly or indirectly connected with glutamine synthesis through glutamic acid. An important point of view is that proposed by Phang JM et al.[[87]], that underscore that at the center of glutamine, ornithine and proline metabolism there

is an intermediate common to all three amino acids, delta-pyrroline 5 carboxylic acid (P5C), critically dependent on proline and proline oxidase (POX also known as PRODH, proline de-hydrogenase) for being produced so generating ATP into mitochondria. Re-cycling of P5C to proline by P5C reductase out of mitochondria where P5C is produced, is indispensable for generating reduced NADP, thus fueling pentose phosphate cycle (PPP), and activating syntheses of pentoses from glucose 6 phosphate with NADPH generation, useful for membrane lipid syntheses. When proline is available, POX/PODH increases degradation of proline sequentially first to glutamate and then to αKG, and POX/PODH dependent production of αKG seems crucial in down regulation of HIF-1 signaling in cancer, therefore it is not surprising that in many cancer POX/PRODH is suppressed, while is up-regulated by the tumor suppressor p53 [88].

Of notice, αKG, more than being just an intermediate of citric acid cycle by αKG-dehydrogenase yelding 6 ATP and generating NADH, is the key molecule for malate - αKG - aspartate shuttle, it is an indispensable cosubstrate for transaminase generating oxaloacetate, allows aspartate to glutamate conversion and indirectly glutamine synthesis, and further has multiple regulatory roles , from being indispensable substrate for prolyl hydroxylases mediating ubiquitination and proteasomal degradation of hypoxia –inducible factor 1 α (HIF-1α), and if not oxidized to succinate may increase DNA and histone hypermethylation [8]. In any case, cycling P5C produced from proline into mitochondria and reduction back to proline in cytoplasm is considered a way for transferring reducing equivalents generated by oxidation of glucose in the PPP into mitochondria to generate ATP [89]. Therefore, peculiarly interesting in cancer is the metabolic relationship between glutamine and proline, and most significant may be both synthesis of P5C from glutamate and metabolic interconversions of P5C to either proline, or glutamate or ornithine first and then arginine. Since on one side glutamate can provide aKG as substrate for citric acid cycle, thus generating reactive oxygen species and signaling activation to autophagy and apoptosis, on the other side glutamate as P5C may enter into proline cycle and so recycling NADP to NADPH while fueling glucose into PPP [90]. From PPP is generated NADPH necessary also for inducing the c-Myc oncogene controlled transcription, which is dependent on adenosine sensing, and therefore nucleoside biosynthetic path, also dependent on P5C and glutamine, may connect c-Myc activation to tumor metabolism [91,92].

Since most ornithine is derived by cleavage of urea from arginine, and although not being a proteinogenic amino acid, ornithine is a key molecule for maintaining nitrogen clearance through citrulline and back to arginine. Recycling of ornithine to arginine after urea cleavage is a pathway requiring both aspartate (derived from glutamine transamination) and N-acetyl-glutamate to form argininosuccinate. Interestingly, the key enzyme involved, arginino-succinate synthetase (AS-Sinth), regulates directly AMPK activation and lipid syntheses by consuming ATP to AMP, but ornithine is either the starting point for syntheses of polyamines on one side, but also substrate giving origin to P5C and the strictly related glutamic –γ-semialdehyde (GSA) , so ornithine is also a precursor of proline synthesis on the other side. Of notice, arginine may give origin in liver and kidneys to both urea and polyamine through agmatine , via an alternative route to the cycle producing ornithine and urea. Agmatine is a metabolite relatively poorly studied and derived by arginine through arginine decarboxylase, then converted by agmatinase to putrescine and urea, a minor pathway also identified in humans [93].

Also, some link among glutamine and serine metabolic pathways may be found in recent data showing that arginine deprivation in cells deficient in AS-Sinth (i.e: deficient in recycling ornithine to arginine after urea cleavage) either upregulates glutamine utilization into citric acid cycle so increasing mitochondrial activity, and also increases serine synthesis from pyruvate subtracting pyruvate for production of lactate by cytoplasmic glycolysis both by stimulating phospho- glycerate de-hydrogenase (PHGDH) and inhibiting pyruvate kinase isophorm 2 (PKM2, peculiarly expressed by cancer cells) acting at either total protein level and phosphorylation changes, a synergic activity finally resulting in a decreased Wartburg phenotype [54]. Still, whether activation of PKM2 activity could blunt cancer development since it increases mitochondrial oxidative phosphorylation

so promoting ROS production, is debated, although its deletion and inhibition of activity proved to worsen cancer evolution [94].

7. Conclusions

Complexity is high when dealing with metabolism and amino acids, and this is reflected by the variety of protocols used and the different interpretations that findings may suggest when protocols are accurately analyzed. Recent data open new insights into cancer metabolism and a series of puzzling questions. Cancer lives and grows in the same environment of normal cells, but does cancer "eat" according the same needs? Does it follow the same rules of normal cells to be competitive and proliferate?

In vitro studies has recently demonstrated that by largely increasing the EAA/NEAA ratio, or altering the reduced ratio (<1) that is usually found in proteins and biological fluids, blunts cancer development, and, although inadvertently demonstrated by some authors, also in vivo supplying EAA in excess than NEAA by subtracting serine and glycine to growth medium, or pellet in in vivo studies, apparently reduce cancer growth. EAA given in excess of NEAA to cancer cells modify different epigenetic and post-translational targets. EAA in excess of NEAA increase autophagy, inhibit proteasome and PARP-1 cleavage, trigger an apoptotic drive, and this while normal cells are unharmed by those modifications. Since in foods, in human proteins and fluids, NEAA are more abundant than EAA, although it should be tested, supplementing large amounts of EAA would not be the solution to treat cancer, but certainly by reversing EAA/NEAA ratios cancer cells are stressed. EAA in excess of NEAA have unveiled a new specific fragility of cancer metabolism that is worth of being fully explored for the possible future therapeutic solutions this safe procedure may suggest.

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