The manuscript entitled ‘The influence of diets with varying essential/nonessential amino acids ratios on mice lifespan’ evaluates the effect of different proportions of essential and non-essential amino acids on lifespan, body and tissue mass, food and water intake and some blood and urine parameters. The main conclusion of the paper is that the proportions of dietary essential and non-essential amino acids affect lifespan and is an important consideration for diets promoting human health. The authors conclude that NEAAs are detrimental to health and should be avoided.

While the topic is interesting, in my opinion, the conclusions are overstated, the results are over interpreted and the experimental design is not sufficient to answer any of the multiple questions posed. It is well known that removing an essential nutrient (also including single amino acids, e.g. leucine) leads to early death in mice. This has been shown many times.

The reference diet is not an appropriate control for any of the experimental diets. The experimental diets use either free amino acids, free amino acids from casein or whole casein protein. The reference diet contains protein from mixed sources with an undefined EAA/NEAA ratio. Furthermore, the experimental conditions are not physiological or achievable (in a human context) and therefore provide little insight into the question of optimal dietary amino acid content.

First of all, we are very grateful to all referees for the attention to our study and data. We peculiarly thank them for observations, suggestions and helpful remarks. We have fully revised and practically re-written text in any part.

We agree with the statement that removing a single essential nutrient, as any single essential amino acid, leads to early death in mice. The use of casein, a reference proteins for mice but lacking sufficient amounts of sulphur containing amino acids (methionine and cysteine/cystine). Accordingly, we have changed, and hopefully simplified identification of the different diets, and peculiarly, we have focused attention on essential amino acids content of diets: 0%, 30%, about 55% (Standard laboratory diet, casein as whole protein and casein under form of free amino acids), 100% amino acids. This latter is a peculiarly sophisticated one, since it provides either sulphur containing amino acids providing both methionine and cystine/cystine in a most healthy since balanced ratio according literature: Verhoef P, Steenge GR, Boelsma E, van Vliet T, Olthof MR, Katan MB. Dietary serine and cystine attenuate the homocysteine-raising effect of dietary methionine: a randomized crossover trial in humans. Am J Clin Nutr. 2004 Sep;80(3):674-9.) or tyrosine, which is an indispensable amino acid for any organ but liver, the only organ containing metabolically significant amounts of phenyl-alanine hydroxylase necessary for synthesizing tyrosine from phenyl-alanine.

What we have shown is primarily that life is possible when rodents are fed just by free amino acids. Also, that there is a very short (near 15%) interval among essential amino acids provided by food proteins and diets providing 30% essential amino acids and a death as rapid as that provided by totally essential amino acids free ones. Please notice that the essential amino acids content of EAA30% diets, containing 20 grams of amino acids any 100 grams of pellet, provided 6 grams of essential amino acids. Most literature consider perfectly sufficient diets providing 15g of proteins (15% of pellet weight), and proteins contain approximately a maximum of 45% of essential amino acids, thus about 6.75 grams of essential amino acids any 100 grams of pellet. On the other hand, elimination of non-essential amino acids from diets not only is perfectly compatible with life but even prolongs lifespan, and this is the first evidence of this kind, at our knowledge. Although we have not tested an intermediate value among 45% of essential amino acids and 100% amino acids, a type of diet that now we know would have been interesting to be tested in a full life span, we have already tested on a shorter life span some diet providing 80% essential amino acids and 20% of selected non essential amino acids (Body Weight Loss and Tissue Wasting in Late Middle-Aged Mice on Slightly Imbalanced Essential/Non-essential Amino Acids Diet. Corsetti G, Pasini E, Romani C, Galvani R, Picca A, Marzetti E, Fiati V, Dioguardi FS. Front Med (Lausanne). 2018 May 17;5:136. doi: 10.3389/fmed.2018.00136. eCollection 2018. PMID: 29868589) with results complying with those observed with 100% essential amino acids. The latter, indeed, contains cysteine/cystine (cystine and cysteine are in continuous balance at physiological pH) and tyrosine for the said reasons, and together so providing 4.50% of non-essential amino acids, if those are considered so according a most restrictive point of view.

On those bases, we consider acceptable, if not already proven the hypothesis that more than the amount of essential amino acids, is the ratio among essential amino acids and non-essential amino acids that is indeed related to life-span. But also that primarily is of excess non-essential amino acids that is detrimental to life.
Reviewer ANSWER: I agree with the conclusion that life is possible when completely excluding non-essential amino acids from the diet. However, from the current design, it is not possible to make the conclusion that removing essential amino acids from the diet extends lifespan. The Standard diet contains an undefined amino acid profile. You have extensively discussed in your response the impact that the specific amino acid profile (not just EAA vs. NEAA) can have on lifespan... As such, there is no way of knowing whether the observed increase in lifespan is the result of 1) a lack of NEAA; 2) A high intake of EAA; or 3) an alteration in the intake a specific amino acid. Therefore, an appropriate control group will depend on the question you are trying to answer. If you want to say a lack of NEAA extends lifespan, then you should compare an EAA only diet to a diet containing the same amount of EAA, plus NEAA.

AUTHORS ANSWER: This is an interesting point and suggestions are very wise. Indeed, as suggested by our actual findings, different protocols should be interesting at the purpose to fully evaluate the hypothesis that inversion of the ratio EAA/NEAA usually present in food proteins and < or <=0.9 is connected to prolonged life span. In a paper published in Cell Metab 2010 (citation 11), indeed, we already observed that supplementation to normal diet of our EAA formulation to rats improved lifespan, but this protocol should be considered biased by the different N amount in the two groups, largely superior in EAA supplemented animals, although NEAA intake was the same in the two groups. Thus, the wise Referee poses a pivotal question that needs a complex series of protocols to be answered: is this formulation or any formulation rich in some EAA similarly efficient? Since the quantitative requirement of any single EAA, as we actually know, it is not the same (leucine and tryptophan requirements in grams/kgBW are very different, although linked, as an example), it would took a immense series of different EAA formulations to provide a full answer, if ever this would be possible. I hope that the Referee will agree that at least we establish a small benchmark, but still a benchmark, useful for targeting at best further studies, and that both our detailed EAA and NEAA formulations may be a useful reference to be used by other researchers in any further study. Our EAA-100% based formulation allows life, and a prolonged one. As we know, life based on free EAA was never tested for a full lifespan in mice. At least, its safety is proven. And, if I understand, the Referee agrees that that formulation will prolong life when compared with a standard laboratory diet, but he suggests that we cannot be sure, based on our protocol, that removing NEAA would improve lifespan. But, certainly, by increasing NEAA on EAA, although EAA are given in sufficient amounts to match minimal requirements by the EAA-30% diet, drastically reduces lifespan. We have to observed that by the EAA-30% diet we provide 6 grams of EAA any 100 grams of pellet, and by a standard laboratory diet AIN93, see: Tab 8, pag 1944 of J. Nutr. 123: 1939-1951, 1993, amino acidic composition of a diet containing 14 % of proteins is presented as reference. EAA/NEAA ratio is 0.86, and thus optimal standard EAA requirements are indicated as 46% of 14 grams: in grams per 100 grams of pellet, this is 6.44. And their lifespan is extremely similar to those introducing only NEAA. By AIN93 diet, 7.5 grams of NEAA are given, by EAA-30%, containing 20% of amino acids, 14 grams of NEAA were provided, near twice the AIN93 diet. This is why we hypothesized that NEAA are the main effectors of a shorter life, based on our data.

Reviewer: If you want to conclude that a high amount of EAA prolongs lifespan, then similarly, the NEAA content should not change and you should alter the EAA content. If you want to address the issue of specific EAA:NEAA ratios, then you need to have a control group with the same source of EAA and NEAA, but at different ratios.

AUTHORS ANSWER: The data that we present here, were indispensable to project future studies. If we would find a way to be financed, indeed we would test different diets, but if changing EAA to NEAA ratios while providing the same amount of nitrogen (i.e.: same grams of amino acids by pellets, 20% in our study) was our option, giving the same amount of grams of NEAA (g.e.: 54% of 20 grams of amino acids any 100 grams of pellet, 10.8 grams of NEAA) and increasing proportionally the grams of EAA provided, that is doubling (50/50, 21.6 grams of amino acids) or further increasing (i.e:75EAA/25NEAA, i.e.: a total of 27 grams of amino acids) would increase enormously the total amount of nitrogen intake and percent of total calories provided as amino acids.

For further details, please go to further answer to point 6, were a quantitative example has been provided. This is why, and obviously this study is a beginning of a research path, we decided to maintain constant (20%) the percentage of proteins, or of amino acids, provided by pellets.

Reviewer: In my opinion, the conclusions remain overstated. Especially the last paragraph of the conclusion (line 533-540). This study has been conducted in mice, which are omnivores and are
therefore likely to naturally get higher amounts of EAA from animal sources and lower from plant based sources… The results cannot be used to make a broad conclusion about evolution. Predators would also get a high EAA content from eating meat, so I don’t see how nature is designed to limit their numbers. For herbivores, it is unclear whether the ratio of NEAA:EAA also has the same effect. Regarding casein, a milk protein, ‘mother nature’ also includes whey protein (which is higher in EAAs) in the milk… which hardly seems cruel.

**AUTHORS ANSWER:** We will modify the statement, we will propose it as a possible hypothesis worth of being explored.

Indeed, this is an interesting observation: still we do not think that there is a quantity of EAA that would “save” animals from damages exerted by the contemporary introduction of NEAA in ratios contained in food proteins (< or <<0.9). Most studies dealing with some kind of caloric restriction indicates that EAA restriction reduces body weight (and growth) and allow survival, but the possible benefits of caloric restriction in reducing peculiarly NEAA has never been discussed. Cutting ¼ of 46% EAA (11.5), and ¼ of 54% NEAA (13.5) any 100 grams of proteins ingested by animals, if we hypothesize NEAA as toxic and antagonists of the indispensable EAA effects on epigenetics (g.e: increased EAA/NEAA ratios trigger autophagy, see citation 19, Bonfili et al., as well as glutamine deprivation and activation of glutamine synthesis from EAA, see: http://dx.doi.org/10.4161/auto.22152), would reduce the effects of the intake of NEAA per gram of BW, still matching minimal EAA needs for survival. Our point is not that EAA are beneficial, they are indispensable and allow life, but, on the contrary, that NEAA are detrimental, provided in large excess of EAA, although dispensable (please look also at our study published in Cell Metab 2010, citation 11).

Milk provides more amounts of EAA by yeast protein, a protein providing a ratio of EAA/NEAA near <0.9, than by casein, but yeast protein is present in an extremely minor amount if compared to casein. And no alimentary protein provides an EAA/NEAA ratio > 0.9. This may be linked to the effects of EAA/NEAA ratios > or >> 1, as we have found that by largely increasing EAA ratios WAT virtually disappears. In nature, animals need fat to survive to cold climate and in periods of reduced food availability: apparently, matching EAA with NEAA “feeds” WAT cells and epigenetically condition their development, also an unexpected and important finding of our study. Then, Nature kills preferably the less efficient ones, those that would grow less in conditions similar to those that would be faced during a competitive wild life and a seasonal change in quality of foods, since the beginning. Wild animals indeed live far shortest than captive ones. But, we would modify our conclusions in agreement with Referee’s suggestions.

**Specific comments**

1) **Figure 2D-F:** These are unnecessary graphs. They could all be replaced with a graph showing: g food/ kg body weight/day The graph labels indicate body weight change, but the graph shows absolute body weight. In Figure 2D there is a typo. NEAAR-R.

Thank you for suggestions and help in ameliorating presentation of data and correction of our errors.

2) **There appear to be no statistics performed on the lifespan curves.**

We have now reported statistics on survival rate and relative p < , at IC 0.95, calculated by Mantel-Cox test.

3) **The level of blood hpg in the casein diets are very high, suggesting that the diet is inducing a strong systemic inflammatory response. This is surprising seeing as a majority of protein in many laboratory diets is sourced from casein. It is also very surprising that the casein diet resulted in a dramatically shortened lifespan compared to the Std diet. Are the authors sure that the casein used or the food was not contaminated?**

Yes, we are sure of the safety of our casein, since casein from the same bulk was used to prepare other pellets, and used in other experiments, and therefore of the best standard available. Indeed, referee’s note is very skilled, and linked to the protocol, since pure casein diets induce a qualitative

4) Line 237-238. It is well known that limiting the supply of essential nutrients (even just one amino acid, such as leucine) leads to malnutrition and death. It is inaccurate to describe this as sarcopenia-wasting-cachexia. It is malnutrition.

This is an interesting observation and worth of a peculiar semantic discussion. Indeed, we think that sarcopenia, wasting and cachexia are characterized by a sequence of peculiar biological alterations, perfectly fitting with our observations. And, yes, in our model those modifications are consequent to malnutrition. We think that both statements are correct: we describe a model of sarcopenia, wasting and cachexia dependent on qualitative (and not quantitative) malnutrition.

Reviewer: ANSWER: This is still not sarcopenia. Unless the mice grow and mature normally and then age rapidly, they are not sarcopenic. Cachexia could be appropriate in the context of the casein diet, but for the high NEAA content diets, malnutrition is the most fitting description.

AUTHORS ANSWER: We fully accept this observation: indeed, animals lose muscle mass, but far less lose muscle function until cachexia appears. We will substitute sarcopenia with “muscle losses”, but we kindly ask: sarcopenia, wasting and cachexia may or not be consequent to malnutrition?

5) Line 384-389 – This paragraph does not reflect the content of the manuscript. Please remove.

We have completely revised, and re-written the full paper. We hope to have met the task to be more compliant with referees advice and suggestions.

6) Line 373 - It is well established that calorie restriction extends lifespan. It is also well established that reduced EAA intake extends lifespan. This manuscript relates to malnutrition and not the processes regulating aging.

Some of the authors are very discriminating about calorie restriction, we observe that it is most effective peculiarly in animals whose dimensions grow all lifelong. Efficiency of caloric restriction n primates is still a controversial item, and as an example, calorie restriction in the elderly, indeed, is considered potentially dangerous and life threatening. Reduced essential amino acids intake to promote life span is also a questionable sentence: first of all we show that essential amino acids limitation should not be accompanied by a proportional increase in non-essential amino acids, moreover, even in Caenorhabditis Elegans, both deficiencies and excess of different amino acids may prolong or reduce lifespan (Edwards et al. Mechanisms of amino acid-mediated lifespan extension in Caenorhabditis elegans. BMC Genetics (2015) 16:8. DOI 10.1186/s12863-015-0167-2). We have been warned in comments “that removing an essential nutrient (also including single amino acids, e.g. leucine) leads to early death in mice”, and this should sound contradictory if our study is not taken into account. We give a normal amount of nitrogen as amino acids or proteins, 20% of total food intake, although this amount is in highest percentiles of what is considered a normal intake, and it is possible that increasing those levels may be deleterious.

But, we suggest, and on the bases of the calculations of essential and non essential amino acids content of foods explicated above, that caloric restriction may be efficient, at least in part, due to a reduced total non-essential amino acids intake, because through caloric restriction non-essential amino acids intake is also reduced, and this minimize their toxicity, while body size reduction driven by caloric restriction allows survival by the amount of essential amino acids introduced with the 45% of essential amino acids provided through normal nitrogen intake. Indeed, we show that, at least in rodents, a diet based exclusively on a (well) balanced essential amino acids formulation prolongs lifespan, and this is coupled to a spontaneously reduced caloric intake and increased water intake, reduced body weight and normally developed length.
Supplementation of diets with essential amino acids have been already studied and shown to prolong lifespan better than some kind of caloric restriction (see citation 8 and 17).

On the other hand, we are well aware, as we have tested in another study, that alterations of the ratios among some essential amino acids in the formulations that we have tested is scarcely compliant with a prolonged lifespan, for instance (data not shown, will be published elsewhere).

Reviewer: ANSWER: It is an interesting suggestion that a reduced NEAA intake is the reason for the life-prolonging effects of CR – worth testing in the future! However, you did not test this here. The appropriate experiment would be to leave EAA content the same and then reduce or remove the NEAA content.

Authors Answer: This is a very reasonable suggestion and question, that arise a main problem. In a pellet providing 20% grams of amino acids, and, as an example, 56% are NEAA and 44% EAA, 11.2 grams of NEAA any 20 grams are provided. If we take away 11.2 grams, we have to possibilities: to match 20 grams by substituting NEAA with EAAs (as it was our first choice, and we have explored by the EAA-100% diet), or to test a totally different diet providing only 8.8 grams of amino acids (8.8% of total calories) to one providing more than twice nitrogen, and compensating the 11.2 % grams of amino acids by carbohydrates and lipids. We do not have any idea of how this kind of selective “qualitative” caloric restriction would have influenced lifespan, but exploring this diet would have introduced a further complication in an already complex study.

7) the reference diet used (Std) is not an appropriate control.

We have difficulties in understanding this observation. We cannot think to a different diet than standard diet, fully compliant with what prescribed by Reeves PG at al. AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J. Nutr. 123: 1939-1951. (1993), and that thus should be considered a normal laboratory diet suitable to be uses as standard control in a lifespan study.

Reviewer: ANSWER: See point above. It is a great standard diet… so long as the manipulations to the diet at still comparable. You are comparing an AIN93 diet containing protein (of an unknown AA profile) with AIN93 without protein, plus AAs of a known profile. The correct control diet would be AIN93 without protein, plus AAs of the same profile at the same EAA:NEAA ratio as normal AIN93.

Authors Answer: Now I got the point, thanks. This is a very correct observation. But we had no different choices. We were (and still we are) not able to measure by ourselves the amino acidic composition of standard laboratory diets provided by different well acknowledged producers, and although we tried to have those data from them, they denied the information as not available, and warned that from one batch to another amino acidic composition may have varied since the source of fish proteins may have been different, and only nitrogen content as established by a simple Lawry method would have been carefully monitored and kept constant at production sites. This lack of information, and the possible biases in prolonged observational studies linked to differences in amino acidic composition provided by different protein sources is a main point established by this study. Indeed, we cannot trust homogeneity of amino acids formulations provided by nitrogen composition of batches produced in different times of commercial pellets, although their use is a standard procedure. In the future, we would plan to prepare our own standard pellet by using always the same controlled bulk proteins, although aware that we would have great difficulties in establishing a precise amino acid profile of proteins. The only chance we had was to compare a single protein to its amino acidic composition. This is why we had chosen casein, and reproduced as well as we could its amino acidic composition (even amino acidic compositions of casein found in literature was not the same according all authors). But, a discrete variety in food composition is what has to be faced in real life, and not present in monotonous diets provided to animals in this study. We agree and are aware that biases are enormous, in any lifespan dealing with food. But this is a start, and we hope that even the most helpful, although critical Referee had to admit that this study, with all its limits, somehow stimulated his mind and would influenced his future way of looking at influences of nutrition and lifespan.