In Vivo Assessment of Glutamine Anaplerosis into the TCA Cycle in Human Pre-malignant and Malignant Clonal Plasma Cells

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***In Vivo* Assessment of Glutamine Anaplerosis into the TCA Cycle in Human Pre-malignant and Malignant Clonal Plasma Cells**

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- 25 - Glutamine anaplerosis into the TCA cycle is relatively higher in malignant bone marrow
26 plasma cells compared to pre-malignant plasma cells.
- 27 - Quantitative levels of TCA cycle intermediates differentiate between MGUS and MM bone
28 marrow microenvironments while reflecting their clonal plasma cell burden.

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30 **Key words:** stable isotope metabolomics, plasma cell malignancies, myeloma, glutamine

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ABSTRACT

Background: Overexpression of c-Myc is required for the progression of pre-malignant plasma cells in monoclonal gammopathy of undetermined significance (MGUS) to malignant plasma cells in multiple myeloma (MM). c-Myc also increases glutamine anaplerosis into the tricarboxylic acid (TCA) cycle within cancer cells. Whether increased glutamine anaplerosis is associated with the progression of pre-malignant to malignant plasma cells is unknown.

Methods: Human volunteers (N = 7) and patients with MGUS (N = 11) and MM (N = 12) were prospectively recruited to undergo an intravenous infusion of ^{13}C -labelled glutamine followed by a bone marrow aspiration to obtain bone marrow cells and plasma.

Results: Despite notable heterogeneity, stable isotope resolved metabolomics (SIRM) revealed that the mean ^{13}C -labelled glutamine anaplerosis into the TCA cycle was higher in malignant compared to pre-malignant bone marrow plasma cells relative to the remainder of their paired bone marrow mononuclear cells. RNA sequencing demonstrated a higher relative mRNA expression of c-Myc and glutamine transporters such as ASCT2 and SN2 in malignant compared to pre-malignant bone marrow plasma cells. Finally, higher quantitative levels of TCA cycle intermediates in the bone marrow plasma differentiated MM from MGUS patients.

Conclusion: Measurement of the *in vivo* activity of glutamine anaplerosis into the TCA cycle provides novel insight into the metabolic changes associated with the transformation of pre-malignant plasma cells in MGUS to malignant plasma cells in MM.

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INTRODUCTION

Multiple myeloma (MM) is the second most common hematological malignancy in the US with over 20,000 new MM patients being diagnosed each year.[1] This malignant plasma cell disorder is associated with devastating end-organ damage such as bone destruction, renal failure, anemia, and hypercalcemia.[2] Despite improvements in survival with the advent of novel therapies[3-5], MM remains mostly incurable with a median survival of only 5 to 7 years.[6] Thus, identifying novel targets in clonal plasma cells (cPCs) from MM that can be exploited for clinical purposes either for early diagnosis or therapeutic purposes remain critical.

Malignant cells have altered cellular metabolism in order to meet increased requirements for nutrients and energy.[7] Although up regulation of aerobic glycolysis (the “Warburg effect”) is common in most cancer cells, it is insufficient to support their increased anabolic metabolism.[8] The energy/nutrient deficit is overcome mostly by the tricarboxylic acid (TCA) cycle whereby TCA intermediates serve as precursors for the biosynthesis of fatty acids, nucleic acids, and proteins, and need constant replenishment.[9] Glutamine anaplerosis or entry into the TCA cycle[10] via the formation of glutamate from glutamine and the subsequent formation of alpha-ketoglutarate from glutamate is one method of replenishing the TCA cycle intermediates.[11]

Overexpression of the transcription factor c-Myc is required for the pathogenesis of malignant plasma cells in MM from their precursor state of pre-malignant plasma cells in monoclonal gammopathy of undetermined significance (MGUS). *c-Myc* activation signatures are mostly absent in pre-malignant plasma cells derived from patients with MGUS, whereas 70% of new MM patients have up-regulated *c-Myc* activation signatures in their malignant

plasma cells.[12] Furthermore, *Myc* activation in germinal center B cells leads to formation of sporadic MM tumors in *Vk*MYC* mice suggesting its critical role for the progression into MM.[13] Interestingly, c-Myc also promotes transcription of glutamine transporters ASCT2 and SN2 on cell membranes [14] and upregulates GLS1 protein expression to increase conversion of glutamine to glutamate by suppressing miR23a/b (both being negative regulators of GLS).[15] Thus, c-Myc leads to increased uptake of glutamine in cells and also increases the conversion of glutamine into glutamate which then enters into the TCA cycle (i.e. glutamine anaplerosis) in mitochondria.[16] However, it remains to be determined whether the level of glutamine anaplerosis into the TCA cycle is higher in malignant plasma cells of the bone marrows of MM patients relative to their adjacent bone marrow mononuclear cells is unknown. And if so, it is uncertain if increasing glutamine anaplerosis activity into the TCA cycle is associated with the progression of pre-malignant plasma cells in MGUS to malignant plasma cells in MM.

There is a gap in knowledge of the activity of metabolic pathways in pre-malignant plasma cells from MGUS due to the lack of adequate *in vitro* models, as human myeloma cell lines (HMCLs) only represent malignant plasma cells from patients with end-stage MM. In addition, cell culture artifacts from *in vitro* approaches remain a major barrier in the study of tumor metabolism in humans.[17] To overcome these barriers, this study compared the activity of glutamine anaplerosis into the TCA cycle in pre-malignant plasma cells and malignant plasma cells obtained from MGUS and MM patients respectively to their paired remainder of the bone marrow mononuclear cells by using *in vivo* stable isotope-resolved metabolomics (SIRM) to trace the incorporation of ¹³C glutamine into the intermediates of the TCA cycle.

RESULTS

Glutamine anaplerosis into the TCA cycle is present in human myeloma cell lines.

The HMCLs, RPMI-8226 and MM1S, like most cancer cell lines require glutamine supplementation in the cell culture media for optimal proliferation and survival. Our prior study demonstrated glutamine as being the preferred supplier of carbon substrate for the TCA cycle intermediates via its anaplerosis into the TCA cycle in comparison to glucose oxidation when assessed over a time period of 12 hours.[18] The expected isotopomer labeling pattern of TCA cycle intermediates when [$^{13}\text{C}_5$]-glutamine undergoes anaplerosis into the TCA cycle is depicted in **Supplementary Figure 1**. However, this incorporation of ^{13}C into the TCA cycle intermediates in HMCLs is rapid as was observed when RPMI-8226 and MM1S HMCLs were cultured for only 60 minutes in RPMI-1640 media containing 2 mM of glutamine of which only 5% was enriched with [$^{13}\text{C}_5$]-glutamine (**Supplementary Figure 2**). This rapid uptake of glutamine is not unexpected given the very high proliferation and short doubling time of HMCLs that require nutrients like glutamine for biomass, energy and redox homeostasis. However, recent studies have demonstrated that the extent of a cancer cell line's dependence on glutamine anaplerosis may differ based on its environmental context such as the level of cystine.[19] Thus, whether our *in vitro* observation of glutamine anaplerosis into the TCA cycle of HMCLs is observed in humans *in vivo* in bone marrow pre-malignant and malignant plasma cells and their paired remainder of the bone marrow mononuclear cells is unclear.

Intravenous infusion of ^{13}C -labelled glutamine in human volunteers is feasible, safe and subsequently detectable in non-malignant bone marrow plasma cells

A total of seven healthy volunteers were prospectively recruited to participate in a pilot study evaluating the feasibility and safety of assessing the *in vivo* utilization of glutamine by their bone marrow plasma cells. All volunteers, in the fasting state, received an infusion of [$5\text{-}^{13}\text{C}$]-glutamine as a bolus dose over 5 minutes followed by a continuous infusion of this agent over 60 minutes prior to undergoing a bone marrow aspiration to harvest bone marrow plasma cells with the infusion ending immediately thereafter (**Figure 1A**). The clinical characteristics of these seven volunteers, the bolus and continuous infusion dosages of $5\text{-}^{13}\text{C}$ -glutamine infused and their pre- and post-infusion serum ammonia and blood urea nitrogen levels which were measured to assess for metabolic toxicities are listed in **Table 1**. The infusion generated a persistent ($m + 1$) glutamine enrichment in the peripheral blood ranging from 4% – 10% within the first fifteen minutes of the continuous infusion (**Figure 1B**). The $5\text{-}^{13}\text{C}$ -glutamine enrichment was relatively higher in bone marrow plasma compared to paired peripheral blood plasma after 60 minutes of the $5\text{-}^{13}\text{C}$ -glutamine infusion (**Figure 1C**). We next performed a comparison of sorted CD138+ (non-malignant plasma cells) and paired CD138- cells (remainder of the bone marrow mononuclear cells) for the enrichment of ^{13}C in their intracellular TCA cycle intermediates. The cell sorting process for bone marrow mononuclear cells based on CD138 expression of four of the seven volunteers were inadequate for analysis due to technical processing errors incurred initially in this study. Thus, sorted cells were available only in three volunteers, and the enrichment of ^{13}C in their intracellular TCA cycle intermediates was normalized to the steady state ^{13}C ($m + 1$) glutamine enrichment in their respective bone marrow plasma samples. **Figure 1D**

shows the observed enrichment of ^{13}C in the intracellular intermediates from the first turn of the TCA cycle in CD138+ plasma cells and their paired CD138- bone marrow mononuclear cells. This demonstrated the feasibility of the *in vivo* SIRM methods for assessing glutamine anaplerosis into the TCA cycle in bone marrow plasma cells and the remainder of their paired bone marrow mononuclear cells.

Glutamine anaplerosis into the TCA cycle in pre-malignant and malignant plasma cells relative to their paired bone marrow mononuclear cells

We next evaluated the amount of glutamine anaplerosis into the TCA cycle of pre-malignant and malignant plasma cells from MGUS and MM patients respectively relative to the remainder of their paired bone marrow mononuclear cells. A total of eleven MGUS and twelve MM patients were prospectively enrolled on this study. To improve the mass-spectrometry (MS) detection of the various TCA cycle isotopologues of interest, we used an intravenous infusion of [$^{13}\text{C}_5$]-glutamine (i.e. as it would form m+5 and m+4 isotopomers in the first turn of the TCA cycle) instead of the previously utilized 5- ^{13}C -glutamine (which forms m+1 isotopomers in the first turn of the TCA cycle). [$^{13}\text{C}_5$]-glutamine was administered as a bolus dose (4 mg/kg) over 5 minutes followed by a continuous infusion (4 mg/kg/hr) over 60 minutes prior to undergoing a bone marrow aspiration to harvest bone marrow plasma cells with the infusion ending immediately thereafter. The clinical characteristics of these MGUS and MM patients and their corresponding bolus and continuous infusion dosages of [$^{13}\text{C}_5$]-glutamine are listed in **Table 2 and Table 3** respectively. The infusion generated a (m+5) glutamine enrichment ranging from 3% – 7% in the bone marrow plasma after completing 60 minutes of the continuous infusion which was similar between MGUS

185 and MM patients (**Figure 2A**). Upon acquiring the bone marrow aspirate sample, subsequent
186 processing was performed to yield bone marrow CD138+ cells (pre-malignant or malignant
187 plasma cells) and paired CD138- (remainder of the bone marrow mononuclear cells) from
188 each aspirate sample. The cell sorting process for bone marrow mononuclear cells based on
189 CD138 expression yielded sufficient clonal pre-malignant plasma cells in only ten of the
190 eleven MGUS patients and sufficient clonal malignant plasma cells in only eleven of the
191 twelve MM patients. As a result, isotopomer data evaluating the incorporation of ^{13}C from
192 glutamine into the TCA cycle intermediates of CD138+ and their paired CD138- cells was
193 available for assessment in only ten MGUS and eleven MM patients (**Figure 2B**). The mean
194 ^{13}C labelling of the TCA cycle intermediates (glutamate, citrate, fumarate, malate and
195 aspartate) derived from [$^{13}\text{C}_5$]-glutamine anaplerosis into the first turn of the TCA cycle were
196 higher in CD138+ malignant plasma cells from MM patients compared to the remainder of
197 their paired CD138- mononuclear cells. In contrast, the mean ^{13}C labelling of the TCA cycle
198 intermediates derived from [$^{13}\text{C}_5$]-glutamine anaplerosis into the TCA cycle were not
199 significantly higher in CD138+ pre-malignant plasma cells from MGUS patients compared to
200 the remainder of their paired CD138- mononuclear cells except for the metabolite aspartate.
201 To better understand the observed differences in the mean ^{13}C labelling of the TCA cycle
202 intermediates between the two disease groups, the ^{13}C labelling of individual TCA cycle
203 intermediates were also evaluated in each individual MGUS and MM patient by assessing
204 their fractional ^{13}C enrichment (i.e. ratio of ^{13}C enrichment of a TCA metabolite in CD138+
205 cells/ CD138- cells such that a ratio of > 1 reflects higher ^{13}C labelling of the specific
206 metabolite in CD138+ cells). This analytical approach demonstrated notable variability in
207 the fractional ^{13}C enrichment of individual TCA cycle intermediates within pre-malignant

and malignant CD138+ plasma cells obtained from MGUS and MM patients respectively (Figure 2C). Thus, the glutamine anaplerosis into the TCA cycle based on the mean ^{13}C labelling of the TCA metabolites was higher in malignant than pre-malignant CD138+ bone marrow plasma cells when compared to the remainder of their paired CD138- bone marrow mononuclear cells. However, this increased glutamine anaplerosis into the TCA cycle in CD138+ malignant plasma cells compared to the remainder of their paired CD138- mononuclear cells was not uniformly observed in all MM patients. On the contrary, it was also observed even in few MGUS patients and their CD138+ pre-malignant plasma cells when compared to the remainder of their paired CD138- mononuclear cells suggesting significant heterogeneity in the activity of this pathway among the pre-malignant and malignant CD138+ plasma cells from MGUS and MM patients respectively. We don't have sufficient follow-up data to assess whether those MGUS patients who demonstrated increased fractional ^{13}C enrichment of the TCA cycle intermediates in their CD138+ pre-malignant plasma cells when compared to the remainder of their paired CD138- mononuclear cells were more likely to develop MM sooner compared to the remainder of the MGUS patients.

Transcriptomic analysis of the expression of c-Myc and glutamine transporters on cell membranes in pre-malignant or malignant plasma cells and their paired remainder of bone marrow mononuclear cells

Transcriptional upregulation of genes involved in glutamine anaplerosis is one of the possible explanations for the differences in glutamine anaplerosis activity to exist between any two groups if present. Our *apriori* hypothesis was that increased *c-Myc* expression leads to the progression of pre-malignant plasma cells in MGUS to malignant plasma cells in MM

with a resultant increase in glutamine anaplerosis into the TCA cycle that is facilitated by the overexpression of GLS1 that converts glutamine to glutamate as well as by overexpression of the glutamine cell membrane transporters ASCT2 and SN2. Therefore, we examined the RNA expression profile of CD138+ pre-malignant and malignant plasma cells as well as their paired CD138- mononuclear cells from the bone marrows of MM and MGUS patients using RNA sequencing. Sufficient RNA was obtained from six patients with MGUS and ten patients with MM. A total of 2300 genes were determined to be differentially expressed between the malignant and pre-malignant CD138+ plasma cells from MM and MGUS groups respectively of which 799 genes were overexpressed and 1501 were under expressed. Hierarchical clustering analysis was performed demonstrating the overall differential expression of genes between the two groups (**Figure 3A**). As expected, there was an increased relative expression of c-Myc mRNA in CD138+ malignant plasma cells from MM compared to CD138+ pre-malignant plasma cells from MGUS as well as an increase in the mRNA expression of glutamine importers such as ASCT2 and SN2 (**Figure 3B**). However, the expression of GLS1 was similar between the CD138+ malignant and pre-malignant plasma cells. There was also an increased relative mRNA expression of c-Myc, GLS1, ASCT2 and SN2 in CD138+ malignant plasma cells compared to their paired CD138- mononuclear cells in MM patients supporting the previous observation of a higher mean ¹³C labelling of the TCA cycle intermediates (glutamate, citrate, fumarate, malate and aspartate) in CD138+ malignant plasma cells from MM patients compared to their paired CD138- mononuclear cells. Interestingly, the relative mRNA expression of c-Myc, GLS1 and ASCT2 in CD138+ pre-malignant plasma cells was also higher than their paired CD138- mononuclear cells in MGUS patients although not to the same extent as seen in MM patients (**Supplementary Table 1**). This suggests that even at

the MGUS stage, there may be a preferential utilization of glutamine by CD138+ pre-malignant plasma cells over their paired CD138- mononuclear cells. It also supports the previous observation of higher fractional ^{13}C enrichment of the TCA cycle intermediates in the CD138+ pre-malignant plasma cells from individual MGUS patients compared to their paired CD138- mononuclear cells.

Quantitative levels of TCA cycle intermediates in the bone marrow plasma of patients with MGUS and MM

Though heterogenous, since the average *in vivo* activity of glutamine anaplerosis into the TCA cycle was different between CD138+ malignant plasma cells from MM patients and their paired CD138- bone marrow mononuclear cells, we assessed the absolute quantification of the concentrations of these TCA cycle intermediates in the bone marrow and peripheral blood plasma of these MM patients and compared them to those from the MGUS patients. Interestingly, there were significantly higher levels of glutamate and aspartate and lower levels of glutamine in the bone marrow plasma of MM patients compared to that obtained from MGUS patients (**Figure 4A**). In contrast, there were no significant differences in the levels of TCA cycle intermediates in the peripheral blood plasma of MM patients compared to MGUS patients (**Figure 4B**). One likely explanation for this difference is that the bone marrow plasma is the closest to representing the surrounding microenvironment of the pre-malignant and malignant CD138+ plasma cells as well as their CD138- mononuclear cells unlike peripheral blood plasma. It is possible that either the intracellular differences in the levels of the TCA cycle intermediates between pre-malignant and malignant bone marrow CD138+ plasma cells or the differences between the CD138- bone marrow mononuclear cells

from MGUS and MM patients are more likely to be captured from extracellular assessments in the bone marrow plasma rather than the peripheral blood plasma. Interestingly, in a cross comparison assessment of the bone marrow plasma concentrations of glutamate and aspartate between the previously described human volunteers and the patients with MGUS and MM, the levels of glutamate and aspartate in MGUS patients was akin to that observed in the human volunteers which together were both lower than that detected in MM patients (**Figure 4C**). Furthermore, the levels of both these TCA cycle metabolites directly correlated to the percentage of CD138+ plasma cells present in their bone marrows (**Figure 4D**).

Finally, among the MM patients enrolled on this study, there were two patients (MM#2 and MM#7) who had distinct clinical phenotypes. The first patient (MM#2) had relapsed/refractory primary plasma cell leukemia with an extensive amount of bone marrow infiltration with malignant plasma cells as well as a high number of circulating malignant plasma cells (**Figure 5A**). Interestingly, this patient had the lowest (m+5) glutamine enrichment in the bone marrow plasma at ~2.85% compared to the remainder of the MM patients despite receiving the same weight-based dose of the stable isotope infusion. A possible explanation could be that the large number of malignant plasma cells consumed most of the intravenously infused U-¹³C-glutamine, thus preventing the bone marrow plasma enrichment from getting to higher level as seen with most of the other MM patients. The second patient (MM#7) had relapsed/refractory MM but all the malignant plasma cells were confined to his chest and mediastinum with no bone marrow involvement in the posterior iliac crest since diagnosis typically seen with the other MM patients (**Figure 5B**). This patient also had the second lowest (m+5) glutamine enrichment in the bone marrow plasma after MM#2 at ~3.35% compared to the remainder of the MM patients. Again, a possible

explanation could be that the presence of several large plasmacytomas comprised of malignant plasma cells in the chest consumed a lot of the intravenously infused U-¹³C-glutamine preventing the bone marrow plasma enrichment from getting to a higher level. Furthermore, the concentrations of the TCA cycle intermediate such as glutamate, succinate, fumarate, malate and aspartate were markedly higher in the bone marrow plasma of MM#2 compared to the remainder of the MM patients likely reflecting the extensive malignant plasma cell burden in the posterior iliac crest site of the bone marrow aspiration (**Figure 5C**). In contrast, the concentrations of the TCA cycle intermediate such as glutamate, succinate, and aspartate were markedly lower and the concentration of glutamine was markedly higher in the bone marrow plasma of MM#7 compared to the remainder of the MM patients likely reflecting the absence of any malignant plasma cells in the posterior iliac crest site of the bone marrow aspiration.

DISCUSSION

The current study demonstrated the *in vivo* activity of glutamine anaplerosis into the TCA cycle in pre-malignant and malignant bone marrow plasma cells from patients with MGUS and MM respectively. Here we show that evaluating the differences between the intracellular metabolism of malignant plasma cells and their paired non-malignant mononuclear cells in the bone marrow is feasible and can offer valuable insight into the metabolic transformation of pre-malignant plasma cells in MGUS to malignant plasma cells in MM. Furthermore, this study also demonstrated that the concentrations of TCA cycle metabolites in the extracellular space such as the bone marrow plasma which is akin to the microenvironment reflect the differences between the intracellular metabolism of malignant and pre-malignant plasma cells or possibly even between the remainder of their paired CD138- mononuclear cells.

While the glutamine anaplerosis into the TCA cycle based on the mean ^{13}C labelling of the TCA metabolites was higher in malignant compared to pre-malignant CD138+ bone marrow plasma cells relative to the remainder of their paired CD138- bone marrow mononuclear cells (**Figure 2B**), this was heterogeneously observed among individual MGUS and MM patients (**Figure 2C**). There were some MGUS patients whose CD138+ pre-malignant plasma cells had higher fractional ^{13}C labelling of their TCA metabolites when compared to the remainder of their paired CD138- mononuclear cells. It would be intriguing if these aforementioned MGUS patients would have a shorter time to progression to MM, however the study follow-up period is not currently sufficient enough to assess this possibility. Nevertheless, this *in vivo* SIRM approach to assess the activity of other metabolic pathways of interest in addition to glutamine anaplerosis in bone marrow plasma cells remains promising. This is even more applicable in smoldering multiple myeloma (SMM), a more

advanced, asymptomatic clonal plasma cell disorder associated with a higher tumor burden than MGUS, but without the end-organ damage seen in MM.[20] Unlike MGUS, where the risk of progression to MM is 1% per year, the risk of progression in SMM is heterogeneous and decreases with time (~10%/year for the first 5 years, ~3%/year for the next 5 years, and ~1%/year thereafter).[21] This suggests that SMM is a mix of patients with pre-malignant plasma cells as in MGUS and patients with malignant plasma cells as in MM but who have not yet developed end-organ damage. Some patients with SMM rapidly progress to MM within one to two years of their diagnosis, suggesting that their plasma cells were already malignant and had biologically progressed to MM.[22] However, at diagnosis the stratification of SMM patients is not obvious given the lack of end-organ damage (e.g., bone destruction or renal failure), and no reliable clinical or pathological methods are available to differentiate between pre-malignant plasma cells in MGUS and malignant plasma cells in MM. Adding ^{13}C SIRM profiling of intracellular metabolic pathways to assist in differentiating pre-malignant from malignant plasma cells could bridge this gap and enable early identification of SMM patients at high risk of progression. Clinical benefit of early therapy in SMM patients likely to progress is well documented.[23] Conversely, identification of those SMM patients at low risk of progression to MM reduces anxiety and prevents risk of toxicity from unnecessary intervention.

While not unexpected, it was interesting to observe that the relative ^{13}C labelling of the intermediates from the first turn of the TCA cycle in RPMI-8226 and MM1S HMCLs when cultured in media containing 5% enrichment with $^{13}\text{C}_5$ -glutamine for 60 minutes (**Supplementary Figure 2**) was higher than observed in the CD138+ malignant plasma cells from MM patients who had similar levels of $^{13}\text{C}_5$ -glutamine enrichment in their bone

marrow plasma for approximately the same duration (**Figure 2B**). The very high proliferation and short doubling time of HMCLs compared to most primary CD138+ malignant plasma cells from MM patients is likely one explanation for this difference as HMCLs are typically reflective of disease biology that is most aggressive and derived from MM patients at the end stages of their disease. However, another explanation could be that the differences observed in the extent of glutamine utilization between HMCLs and primary CD138+ malignant plasma cells from MM patients may be based on its environmental context such as *ex vivo* versus *in vivo* artifacts assessments.[19] The concentrations of nutrients in cell culture media are not reflective of the true physiological levels experienced by primary CD138+ malignant plasma cells from MM patients.[17] This has major implications when evaluating the potential efficacy of metabolic inhibitors in the pre-clinical setting.

The overexpression of glutamine transporters such as ASCT2 and SN2 observed in malignant CD138+ cells compared to the remainder of their paired bone marrow CD138- mononuclear cells point to their potential as therapeutic targets against glutamine metabolism that are worth investigating in the future in MM models. In fact, Bolzoni et al previously demonstrated the high expression of such glutamine transporters and inhibition of ASCT2 could hinder MM growth in both *in vitro* and *in vivo* models.[24] It would be even more intriguing if these transporters could be modulated to prevent the progression of precursor conditions like MGUS to MM. Finally, it was interesting to observe that the mean ¹³C labelling of aspartate derived from [¹³C₅]-glutamine anaplerosis into the TCA cycle was significantly higher in both CD138+ pre-malignant and malignant plasma cells from MGUS and MM patients respectively when compared to the remainder of their paired CD138-

mononuclear cells. Though not statistically significant, this increased mean ^{13}C labelling of aspartate in the CD138+ cells derived from 5- ^{13}C -glutamine was also observed in the volunteer cohort. This supports prior observations of increased glutamine utilization by the TCA cycle to form aspartate in normal human polyclonal plasma cells and is thought to be important in supporting their function of antibody production.[25] Thus, it is likely that since both pre-malignant and malignant CD138+ plasma cells are constantly producing excess monoclonal antibodies, their utilization of glutamine for aspartate production is equally higher than the remainder of their paired CD138- mononuclear cells.

There are several limitations to this human study. First, it is not known if isotopic steady state was reached in the fraction of CD138+ and CD138- cells from the bone marrow as this would require multiple sampling of bone marrow during the infusion which was not feasible. However, we not only samples similar time points following isotope infusion but also the analytical approach we used here do not require isotopic steady state as we determined the precursor product relationship by normalizing isotopic enrichment of citric acid metabolites to plasma glutamine enrichment. Second, there is likely significant attrition of the ^{13}C labelling within the fraction of CD138+ and CD138- cells from the bone marrow sample as it undergoes various cell sorting processes. However, it is likely that the tracer and tracee loss occurred similarly such that the enrichment values are indicative of the incorporation of glutamine (via glutamate) into the TCA cycle pathway. Moreover, the normalization procedure of the ^{13}C labelling fractions to the enrichment of the stable isotope in the bone marrow plasma as well as relative comparisons of the CD138+ fractions to their paired CD138- fractions allow for adequate comparisons between different groups like MGUS and MM. Despite these limitations, this study demonstrates the feasibility of gaining insight into

the metabolic underpinnings of MM by evaluating the activity of intracellular metabolic networks in malignant plasma cells and their pre-malignant pre-cursors in the bone marrow via *in vivo* stable isotope tracing methodologies. Similar human studies utilizing SIRM methods have been performed in various cancers like lung cancer[26-29], kidney cancer[30] and glioblastoma[31] utilizing a variety of different stable isotope tracers to assess intracellular metabolic pathways. In the future, larger studies may not only be undertaken to assess the *in vivo* measurement of glutamine anaplerosis or even other metabolic pathways as a marker of conversion of MGUS or SMM to MM but also to assess potential effects of molecules that promise to arrest the progression of MGUS or SMM to MM.

METHODS

Research protocol for bone marrow assessment of human volunteers and patients with PC disorders

All methods carried out in this study were in accordance with relevant guidelines and regulations. Approval for this study (IRB#: 17-00313) was obtained from the Mayo Clinic IRB in accordance with the federal regulations and the principles of the Declaration of Helsinki. Prospective human volunteers were recruited following local advertisements under the clinical trial NCT03384108. Whereas consecutive patients with MM and MGUS were prospectively recruited in the clinic under the clinical trial NCT03119883. The diagnostic criteria of International Myeloma Working Group (IMWG) were applied to confirm the diagnosis of MGUS and MM. Upon accrual on either study, participants underwent admission to the clinical research and translational unit for performance of the study procedures. The study procedures involved peripheral blood collections at baseline. The next step was to undergo a bolus injection followed by a continuous infusion of either 5-¹³C-glutamine (3–4 mg/kg bolus and 3–4 mg/kg/hr continuous infusion) or [¹³C₅]-glutamine (4 mg/kg bolus and 4 mg/kg/hr continuous infusion) in the volunteers and MGUS/MM patients respectively. Serial peripheral blood assessments were performed only in the volunteers every 15 minutes upon the start of the continuous infusion to assess the enrichment of (m +1) glutamine in the peripheral blood. After 60 minutes of receiving the ¹³C-glutamine infusions, a bone marrow aspiration was performed on the posterior iliac crest and whole bone marrow aspirate samples were collected in cold EDTA tubes for immediate processing.

Extraction of CD138+ and CD138- mononuclear cells

The freshly obtained bone marrow aspirates from patient underwent Ficoll-Paque gradient separation for plasma processing which was stored for later analysis at -80°C. The remnant cellular component of the bone marrow aspirate underwent red cell lysis using ACK lysis buffer. The clonal PCs were extracted using positive selection by mixing the cells with a CD138 positive selection cocktail and anti-CD138 magnetic-activated cell separation microbeads (ROBOSEP™ cell separation system, StemCell Technologies Inc.) in an automated RoboSep cell separation system. Purity of the sorted clonal PCs was confirmed via light chain restriction using slide-based immunofluorescent method.

RNAseq and data analysis

Total RNA was extracted from cells and the extracted RNAs were evaluated using a Qubit (Thermo Fisher Scientific, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The TrueSeq RNA Exome Kit (Illumina, CA, USA) was used to generate the mRNA-Seq library according to the manufacturer's protocol. Constructed libraries were quantified by BioAnalyzer 2100 system using the D1000 kit (Agilent) and Qubit dsDNA BR Assay kits (Thermo Fisher Scientific). All libraries were pooled and sequenced 101 bp paired-end reads on Illumina HiSeq 4000. FASTQ files were uploaded into Partek Flow software (Partek Inc., MO, USA), and primary QC was performed. The STAR (2.7.3a) aligner was used to align reads to the human reference genome (hg38). After alignment, the final BAM files were quantified using Partek E/M algorithm [32] by Ensembl annotations (Ensembl Transcripts release 92). DESeq2 package [33] was used to normalize data and determine differential expression of RNA-Seq between the groups. The false

discovery rate (FDR) by Benjamin and Hochberg method was used to adjust for comparison. FDR value less than 0.05 and fold change ± 2 at raw P value less than 0.05 were considered as a significant change. Hierarchical clustering was performed on the significantly changed genes based on the Euclidean distance and the average linkage clustering algorithm using Partek Flow software.

Methodology for quantitative assessments of TCA metabolites via GC-MS:

Bone marrow plasma and peripheral blood plasma samples were spiked in 15 of internal solution containing [$^{13}\text{C}_5$]-labeled analytes for 2-HG, glutamate and α -ketoglutarate. The proteins were removed by adding 260 μL of chilled methanol and acetonitrile solution to the sample mixture. After drying the supernatant in the speed vacuum, the sample was derivatized with a solution of O-ethylhydroxylamine in pyridine (20 mg/mL) for 1 hour at 35 $^{\circ}\text{C}$, followed by silylation with MtBSTFA + 1% tBDMCS for 1 hour at 70 $^{\circ}\text{C}$ before it was analyzed on an Agilent 5977B GC/MS under electron impact and single ion monitoring conditions. Concentrations of α -ketoglutarate (m/z 360.2), 2-HG (m/z 433.2), and glutamate (m/z 432.2) were measured against a 12-point calibration curves that underwent the same derivatization.

Gas chromatography-mass spectrometry (GC/MS) based isotopomer analyses

Isotopomer analysis of the intracellular and extracellular TCA cycle metabolites from the HMCL cell pellets and spent media respectively were performed using an Agilent Technologies 5977B GC/MS (Agilent Inc, CA, USA). Splitless injections of 1 μL aliquots of the derivatized extracts were injected onto a DB5-MS capillary column (30 m x 250 μm i.d.,

0.25 μ m film thickness; J&W Scientific, Folsom, CA). The mass spectrometer (MS) was operated under electron impact (EI) conditions with selected ion monitoring (SIM). The temperature of the injector was set at 250°C and the transfer line to the MS at 280°C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC temperature program used was: oven held for 0.5 min at 120°C, then increased to 180°C at 25°C/min, then to 270°C at 6°C/min and finally increased to 325°C at 30 °C/min with a hold time of 1 min. Data were processed using MassHunter quantitative analysis software version B.08.00 build 8.0.598.0 (Agilent Technologies Inc., CA, USA) for integration of peaks and calculation isotopic ratios.

SIM was used to monitor the mole percent enrichment for each analyte, such as the fragment (M0) and all labeled mass isotopomer positions (M1, M2, M3 etc.) up to m+2 above the number of carbons in the molecule backbone. M/z values of M0 were monitored for the following intermediates: lactate (m/z 261.2), fumarate (m/z 287.1), succinate (m/z 289.1), α -ketoglutarate (m/z 360.2), malate (m/z 419.3), citrate (m/z 591.4), 2-HG (m/z 433.2) and glutamate (m/z 432.2). The mass isotopomer distribution of each compound was then corrected for natural abundance using the respective standards.[34] We used an appropriate set of linear simultaneous equations to calculate mole percent enrichment of TCA cycle intermediates to understand glucose dependent or glucose independent glutamine metabolism in myeloma cells.

Statistical Analysis

Fractional abundance of ^{13}C in the various TCA metabolites is expressed as mean $\pm$ standard error mean (SEM). Data were compared and analyzed using the paired t test, and significance was defined as $p < 0.05$. For the quantitative levels of TCA cycle intermediates,

given the non-normal distribution of the data, the differences between groups of interest were compared and analyzed using the non-parametric Mann-Whitney U test, and significance was defined as $p < 0.05$. This analysis was performed by using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

553 **FIGURE LEGENDS**

554 **Figure 1A**

555 Timeline of procedures/evaluations performed on the volunteers during the study day.

556 **Figure 1B**

557 Plasma glutamine enrichment during the 5-¹³C-glutamine infusion in each of the volunteers
558 (N = 7).

559 **Figure 1C**

560 Glutamine enrichment in paired bone marrow and peripheral blood plasma after 60
561 minutes of the 5-¹³C-glutamine infusion from each of the volunteers (N = 7).

562 **Figure 1D**

563 Comparison of mean relative ¹³C fractional enrichment of TCA cycle intermediates in
564 CD138 + and CD138 – cells from the bone marrows of volunteers (N = 3). Error bars
565 represent SEM. n.s. is non-significant by paired t-test.

566 **Figure 2A**

567 Glutamine enrichment in bone marrow plasma after 60 minutes of the [¹³C₅]-glutamine
568 infusion in patients with MGUS (N = 11) and MM (N = 12). Error bars represent SEM. n.s. is
569 non-significant by unpaired t-test.

570 **Figure 2B**

571 Comparison of mean relative ¹³C fractional enrichment of TCA cycle intermediates in
572 CD138 + and CD138 – cells from the bone marrows of MGUS (N = 10) and MM (N = 11).
573 Error bars represent SEM. n.s. is non-significant, * p < 0.05, * * p < 0.01 and *** p < 0.001
574 by paired t-test.

575 **Figure 2C**

576 Relative ¹³C fractional enrichment (CD138 +/CD138-) of TCA cycle intermediates from each
577 MGUS (N = 10) and MM (N = 11) patient.

578 **Figure 3A**

579 Hierarchical clustering analysis on the overall differential expression of genes between
580 MGUS (N = 6) and MM (N = 10) patients.

581 **Figure 3B**

582 Relative differences in mRNA expression of c-Myc, GLS, ASCT2 and SN2 between CD138 +
583 cells from MGUS (N = 6) and MM (N = 10) patients in context of their effect on glutamine
584 transport into the cell.

Figure 4A

Violin plots comparing the median concentrations of different TCA metabolites in the bone marrow plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but ≥ 0.05, n.s. non-significant.

Figure 4B

Violin plots comparing the median concentrations of different TCA metabolites in the peripheral blood plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but ≥ 0.05, n.s. non-significant.

Figure 4C

Violin plots comparing the median concentrations of aspartate and glutamate in the bone marrow plasma between the volunteers (N = 7), MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but ≥ 0.05, n.s. non-significant.

Figure 4D

XY-correlation plots comparing the concentrations of aspartate and glutamate in the bone marrow plasma of patients with MGUS and MM and the percentage of clonal plasma cells in their bone marrow.

Figure 5A

Immunohistochemical staining of the bone marrow for CD138 + clonal plasma cells in MM patient #2 with primary plasma cell leukemia as well as peripheral smear evaluation demonstrating the presence of circulating plasma cells.

Figure 5B

Positron Emission Tomography – Computed Tomography (*PET/CT*) demonstrating the presence of a large plasmacytoma in the right chest wall as well as immunohistochemical staining of the bone marrow for CD138 + clonal plasma cells in MM patient #7 with no marrow involvement.

Figure 5C

Graphical visualization of the concentrations of the TCA cycle intermediate such as glutamate, succinate, fumarate, malate and aspartate in the bone marrow plasma of MM patient #2 with primary plasma cell leukemia and MM patient #7 with respect to that of the remainder of the MM patients (represented by the mean ----, maximum and minimum values).

620 **LIST OF ABBREVIATION**

621 **MGUS:** Monoclonal Gammopathy Of Undetermined Significance

622 **MM:** Multiple Myeloma

623 **SMM:** Smoldering multiple myeloma

624 **TCA:** Tricarboxylic acid

625 **cPCs:** Clonal plasma cells

626 **GLS:** Glutaminase

627 **SIRM:** Stable isotope resolved metabolomics

628 **HMCL:** Human myeloma cell line

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DECLARATIONS

Ethics approval and consent to participate

Approval for this study (IRB#: 17-00313) was obtained from the Mayo Clinic Institutional Review Board in accordance with the federal regulations and the principles of the Declaration of Helsinki.

Consent for publication

This is not applicable. There are no personal identifiers utilized in this manuscript and for MM #2 and MM #7 whose bone marrow and radiographical images are provided are deceased.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

There are no competing interests or conflicts of interest among any of the authors.

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Authors' contributions

W.I.G and K.S.N. designed the study, analyzed the data and wrote the manuscript; J.J. performed the bioinformatics analysis and reviewed the manuscript; D.J. performed the histological analyses in this study and reviewed the manuscript; X. M. P., J.G. and A.B. contributed in performing and coordinating the metabolomics assessments; E.A. and L.E. assisted in data analysis and reviewed the manuscript. T.H. and S.K.K was involved in analyzing the data and reviewing the manuscript.

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Table 1: Characteristics of the volunteers participating and the dosages of 5-¹³C-glutamine infused and their pre- and post-blood plasma urea nitrogen (BUN) and ammonia levels.

Pt #	Age	Sex	Glutamine dose		BUN (mg/dL)		NH ₃ (μmol/L)	
			Prime dose	Infusion dose	Pre	Post	Pre	Post
1	35	F	3 mg/kg	3 mg/kg	15	14	< 10	15
2	24	M	3 mg/kg	3 mg/kg	20	15	12	< 10
3	27	F	3 mg/kg	3 mg/kg	16	16	< 10	< 10
4	29	M	3 mg/kg	3 mg/kg	12	12	15	18
5	24	M	3.5 mg/kg	3.5 mg/kg	16	15	< 10	< 10
6	29	F	4 mg/kg	4 mg/kg	19	13	<10	24
7	52	F	4 mg/kg	4 mg/kg	9	13	< 10	19

Table 2: Clinical characteristics of the MGUS patients and their corresponding bolus and continuous infusion dosages of [$^{13}\text{C}_5$]-glutamine.

Patient #	Age	Sex	M-Spike	Isotype	Glutamine enrichment
MGUS #1	52	F	0.4 g/dL	IgG Lambda	5.21%
MGUS #2	65	M	NQ IgA: 476 mg/dL	IgA Kappa	4.78%
MGUS #3	71	M	1.2 g/dL	IgG Lambda	4.61%
MGUS #4	66	M	0.7 g/dL	IgG Kappa	5.57%
MGUS #5	69	M	1.4 g/dL	IgG Lambda	3.49%
MGUS #6	48	M	1.2 g/dL	IgG Kappa	4.90%
MGUS #7	56	F	1.2 g/dL	IgG Kappa	5.84%
MGUS #8	61	F	NQ IgG: 867 mg/dL	IgG Lambda	6.31%
MGUS #9	71	M	NQ Kappa FLC: 47.5 mg/dL	Free Kappa	6.25%
MGUS #10	67	F	0.6 g/dL	IgG Kappa	5.56%
MGUS #11	56	F	NQ IgA: 464 mg/dL	IgA Kappa	6.00%

NQ: Non-Quantifiable

Table 3: Clinical characteristics of the MM patients and their corresponding bolus and continuous infusion dosages of [$^{13}\text{C}_5$]-glutamine.

Patient #	Age	Sex	M-Spike	Isotype	Glutamine enrichment	Primary Cytogenetics	Prior Lines of Therapy	BMPC%	S-Phase%
MM #1	72	F	1.3 g/dL	IgG Kappa	4.25%	Trisomies	2	20%	1.6%
MM #2	65	F	NQ	NS	2.89%	t(11;14), +1q	4	95%	23.8%
MM #3	71	M	1.5 g/dL	IgG Kappa	5.40%	Trisomies	3	15%	0.7%
MM #4	71	M	0.5 g/dL	IgG Kappa	5.49%	Trisomies, +1q	1	40%	2.6%
MM #5	76	F	NQ Kappa FLC: 47.6 mg/dL	Free Kappa	6.61%	Myc sep, +1q, -17p	2	15%	3.0%
MM #6	58	F	NQ Lambda FLC: 11 mg/dL	Free Lambda	6.80%	IgH sep, -17p	3	100%	8.2%
MM #7	53	M	1.9 g/dL	IgG Kappa	3.35%	N/A	5	0%	N/A
MM #8	69	F	NQ Lambda FLC: 51 mg/dL	Free Lambda	6.63%	Trisomies, +1q	1	20%	4.5%
MM #9	75	M	Lambda FLC: 40.5 mg/dL	Free Lambda	6.45%	Trisomies	4	20%	2.0%
MM #10	71	M	1.9 g/dL	IgA Kappa	6.27%	t(11;14), +1q, -17p	3	50%	1.3%
MM #11	71	M	NQ	NS	5.99%	t(11;14), -17p	2	1%	N/A
MM #12	61	M	1.2 g/dL	IgG Kappa	4.35%	Trisomies	1	5%	1.0%

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824 NQ: Non-Quantifiable; NS: non-secretory; BMPC%: bone marrow plasma cell percentage

Figure 1A: Timeline of procedures/evaluations performed on the volunteers during the study day. PB: peripheral blood assessment

Figure 1B: Plasma glutamine enrichment during the 5-¹³C-glutamine infusion in each of the volunteers (N = 7).

Figure 1C: Glutamine enrichment in paired bone marrow and peripheral blood plasma after 60 minutes of the 5-¹³C-glutamine infusion from each of the volunteers (N = 7).

Figure 1D: Comparison of mean ^{13}C fractional enrichment of TCA cycle intermediates in CD138+ and CD138- cells from the bone marrows of volunteers (N = 3) relative to the glutamine enrichment in their marrow plasma. Error bars represent SEM. n.s. is non-significant by paired t-test.

Figure 2A: Glutamine enrichment in bone marrow plasma after 60 minutes of the $[^{13}\text{C}_5]$ -glutamine infusion in patients with MGUS (N = 11) and MM (N = 12). Error bars represent SEM. n.s. is non-significant by unpaired t-test.

Figure 2B: Comparison of mean ¹³C fractional enrichment of TCA cycle intermediates in CD138 + and CD138 - cells from the bone marrows of MGUS (N = 10) and MM (N = 11) relative to the glutamine enrichment in their marrow plasma. Error bars represent SEM. n.s. is non-significant, * p < 0.05, ** p < 0.01 and *** p < 0.001 by paired t-test.

Individual MM patients

Individual MGUS patients

Figure 2C: ^{13}C fractional enrichment of intermediate upon the first turn of the TCA cycle (CD138+/CD138-) from each MGUS (N = 10) and MM (N = 11) patient. MGUS#5 and MGUS#2 were unable to have their glutamate (m+5) and malate (m+2) assessed due to technical errors during GC-MS assessment and have been not reported.

Figure 3A: Hierarchical clustering analysis on the overall differential expression of genes between MGUS (N = 6) and MM (N = 10) patients.

Figure 3B: Relative differences in mRNA expression of c-Myc, GLS, ASCT2 and SN2 between CD138 + cells from MGUS (N = 6) and MM (N = 10) patients in context of their effect on glutamine transport into the cell.

Figure 4A: Violin plots comparing the median concentrations of different TCA metabolites in the bone marrow plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.1$ but ≥ 0.05 , n.s. non-significant.

Figure 4B: Violin plots comparing the median concentrations of different TCA metabolites in the peripheral blood plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but ≥ 0.05 , n.s. non-significant.

Figure 4C: Violin plots comparing the median concentrations of aspartate and glutamate in the bone marrow plasma between the volunteers (N = 7), MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but ≥ 0.05 , n.s. non-significant.

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Figure 5C: Graphical visualization of the concentrations of the TCA cycle intermediate such as glutamate, succinate, malate and aspartate in the bone marrow plasma of MM patient #2 with primary plasma cell leukemia and MM patient #7 with respect to that of the remainder of the MM patients (represented by the mean ----, maximum and minimum values).

Supplementary Figure 1: Expected mass isotopomer distribution of the intermediates in the first turn of the TCA cycle as a result of glutamine anaplerosis of U-¹³C₅-Glutamine. Each intermediate is represented by the number of carbon atoms in their molecular structure with green circle representing ¹³C and black circles representing ¹²C.

Supplementary Figure 2: The percent intracellular isotopomer distribution of metabolites from the first turn of the TCA cycle in RPMI-8226 and MM1S human myeloma cell lines relative to the U-¹³C-glutamine enrichment in the cell culture media.

Figures

Figure 1

Figure 1A: Timeline of procedures/evaluations performed on the volunteers during the study day. PB: peripheral blood assessment. Figure 1B: Plasma glutamine enrichment during the 5-¹³C-glutamine infusion in each of the volunteers (N = 7). Figure 1C: Glutamine enrichment in paired bone marrow and peripheral blood plasma after 60 minutes of the 5-¹³Cglutamine infusion from each of the volunteers (N = 7). Figure 1D: Comparison of mean ¹³C fractional enrichment of TCA cycle intermediates in CD138+ and CD138– cells from the bone marrows of volunteers (N = 3) relative to the glutamine enrichment in their marrow plasma . Error bars represent SEM. n.s. is non-significant by paired t-test.

Figure 2

A: Glutamine enrichment in bone marrow plasma after 60 minutes of the [13C5]-glutamine infusion in patients with MGUS (N = 11) and MM (N = 12). Error bars represent SEM. n.s. is non-significant by unpaired t-test. B: Comparison of mean 13C fractional enrichment of TCA cycle intermediates in CD138 + and CD138 – cells from the bone marrows of MGUS (N = 10) and MM (N = 11) relative to the glutamine enrichment in their marrow plasma. Error bars represent SEM. n.s. is non-significant, * p < 0.05, ** p < 0.01 and *** p < 0.001 by paired t-test. C: 13C fractional enrichment of intermediate upon the first turn of the TCA cycle (CD138+/CD138-) from each MGUS (N = 10) and MM (N = 11) patient. MGUS#5 and MGUS#2 were unable to have their glutamate (m+5) and malate (m+2) assessed due to technical errors during GC-MS assessment and have been not reported.

Figure 3

A: Hierarchical clustering analysis on the overall differential expression of genes between MGUS (N = 6) and MM (N = 10) patients. 3B: Relative differences in mRNA expression of c-Myc, GLS, ASCT2 and SN2 between CD138 + cells from MGUS (N = 6) and MM (N = 10) patients in context of their effect on glutamine transport into the cell.

Figure 4

A: Violin plots comparing the median concentrations of different TCA metabolites in the bone marrow plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test

where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but > 0.05, n.s. non-significant. B: Violin plots comparing the median concentrations of different TCA metabolites in the peripheral blood plasma between MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but > 0.05, n.s. non-significant. 4C: Violin plots comparing the median concentrations of aspartate and glutamate in the bone marrow plasma between the volunteers (N = 7), MGUS (N = 11) and MM (N = 12) groups. Data was analyzed by Mann-Whitney U test where ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.1 but > 0.05, n.s. non-significant. 4D: XY-correlation plots comparing the concentrations of aspartate and glutamate in the bone marrow plasma of patients with MGUS and MM and the percentage of clonal plasma cells in their bone marrow.

Figure 5

A: Immunohistochemical staining of the bone marrow for CD138+ clonal plasma cells in MM patient #2 with primary plasma cell leukemia as well as peripheral smear evaluation demonstrating the presence of circulating plasma cells. B: Positron Emission Tomography – Computed Tomography (PET/CT) demonstrating the presence of a large plasmacytoma in the right chest wall as well as immunohistochemical staining of the bone marrow for CD138 + clonal plasma cells in MM patient #7 with no marrow involvement. C: Graphical visualization of the concentrations of the TCA cycle

intermediate such as glutamate, succinate, fumarate, malate and aspartate in the bone marrow plasma of MM patient #2 with primary plasma cell leukemia and MM patient #7 with respect to that of the remainder of the MM patients (represented by the mean —, maximum and minimum values).