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The immune benefits of vitamin D₃ supplementation beyond calcium and phosphate maintenance are highly clinically debated. Kidney expression of CYP27B1 is the source of endocrine, circulating 1,25(OH)₂D₃ (active form of vitamin D) that maintains serum calcium and phosphate. 1,25(OH)₂D₃ may also be made by the CYP27B1 enzyme in non-renal cells, like immune cells, in a process driven by cellular availability of 25(OH)D₃ and inflammation. Due to the endocrine nature of 1,25(OH)₂D₃ in circulation, it is difficult to discern between these two sources. We recently created a regulatory deletion model of Cyp27b1 (M1/M21-DIKO) where mice have normal inflammatory-regulated Cyp27b1 expression in non-renal tissues (unlike global Cyp27b1-KO), but no expression within kidney. Here, utilizing on-tissue chemical derivatization and Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging (MALDI-MSI), we investigated the distribution of 1,25(OH)₂D₃ and 25(OH)D₃ in the kidney, liver, spleen, and thymus. MALDI-MSI demonstrated increased 1,25(OH)₂D₃ in non-renal tissues such as the spleen after vitamin D₃ supplementation in M1/M21-DIKO mice. Additionally, from this we found increased Il4 and decreased Tnfa in the spleen after vitamin D₃ supplementation. Taken together, these data demonstrate non-renal production of 1,25(OH)₂D₃ in vivo and provide a consequence of vitamin D₃ supplementation and non-renal 1,25(OH)₂D₃ production in cytokine changes.

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Spatial detection and consequences of nonrenal calcitriol production as assessed by targeted mass spectrometry imaging

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Running Title: Non-renal calcitriol MSI

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Abstract

The immune benefits of vitamin D₃ supplementation beyond calcium and phosphate maintenance are highly clinically debated. Kidney expression of CYP27B1 is the source of endocrine, circulating 1,25(OH)₂D₃ (active form of vitamin D) that maintains serum calcium and phosphate. 1,25(OH)₂D₃ may also be made by the CYP27B1 enzyme in non-renal cells, like immune cells, in a process driven by cellular availability of 25(OH)D₃ and inflammation. Due to the endocrine nature of 1,25(OH)₂D₃ in circulation, it is difficult to discern between these two sources. We recently created a regulatory deletion model of Cyp27b1 (M1/M21-DIKO) where mice have normal inflammatory-regulated Cyp27b1 expression in non-renal tissues (unlike global Cyp27b1-KO), but no expression within kidney. Here, utilizing on-tissue chemical derivatization and Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging (MALDI-MSI), we investigated the distribution of 1,25(OH)₂D₃ and 25(OH)D₃ in the kidney, liver, spleen, and thymus. MALDI-MSI demonstrated increased 1,25(OH)₂D₃ in non-renal tissues such as the spleen after vitamin D₃ supplementation in M1/M21-DIKO mice. Additionally, from this we found increased Il4 and decreased Tnfa in the spleen after vitamin D₃ supplementation. Taken together, these data demonstrate non-renal production of 1,25(OH)₂D₃ in vivo and provide a consequence of vitamin D₃ supplementation and non-renal 1,25(OH)₂D₃ production in cytokine changes.

Introduction

Vitamin D has profound abilities to regulate many different biological processes such as influence the differentiation of skeletal cells, change immune programming, process bile acids, control mineral channels, fight cancer cell proliferation, and promote anti-inflammatory responses (1-4). The amount of vitamin D₃ supplementation needed for these various tasks however has been widely debated as human clinical trials have failed to confirm the effects observed from cell culture and animal studies (5-7). These studies are all complicated by renal production of 1,25(OH)₂D₃ (calcitriol) (8). The ability of the body to regulate calcium (Ca) and phosphate (P) relies on proper functioning of vitamin D metabolism with CYP27B1 making 1,25(OH)₂D₃ and CYP24A1 degrading. CYP27B1 expression in the kidney is a highly regulated, endocrine
hormone-controlled process with parathyroid hormone (PTH) inducing and fibroblast growth factor 23 (FGF23) and 1,25(OH)2D3 suppressing (8). CYP24A1 expression is reciprocally regulated to CYP27B1 in that PTH suppresses and FGF23 and 1,25(OH)2D3 induce (9, 10). The balance of these enzymes in the kidney controls vitamin D metabolism in the body.

Outside of kidney, regulation of CYP27B1 and CYP24A1 are quite different. CYP27B1 expression is not controlled by the endocrine hormones PTH, FGF23, or 1,25(OH)2D3 (10). CYP24A1 retains its ability to be regulated by 1,25(OH)2D3, however, FGF23 and PTH fail to change the expression in non-renal tissues (9). Additionally, the non-renal expression levels of Cyp27b1 and Cyp24a1 are 100- to 1000-fold lower than what is observed in the kidney in mice (9, 10). However, these non-renal cells do retain low and measurable expression of Cyp27b1. Cyp27b1 in these tissues is induced by inflammatory signals like lipopolysaccharides (LPS), IL-1β, and others (11). It has also been demonstrated that macrophages can produce 1,25(OH)2D3 during sarcoidosis in humans (12). Substrate availability (25(OH)D3) for CYP27B1 is key to the physiologic regulation of vitamin D metabolism as 25(OH)D3 bound to an albumin binding protein (vitamin D binding protein, DBP) is transported through the cell surface receptors megalin and cubulin (13-15). These receptors have high expression in the kidney, so the availability of 25(OH)D3 remains high, however, other tissues have far lower expression and thus, for this reason, it is believed they may have lower availability of 25(OH)D3 (16). Additionally, a recent article examining 1,25(OH)2D3 levels in anephric patients found that low levels of circulating 1,25(OH)2D3 were present and this was postulated to be coming from non-renal sources driven by substrate availability (17).

We recently solved the renal regulatory mechanism for Cyp27b1 expression through key upstream enhancers in the proximal convoluted tubule cells (18) in intronic regions of the neighboring Mettl1 and Mettl121b (Eef1akmt3) genes (10, 11). We used CRISPR-Cas9 editing to create a mouse (M1/M21-double intronic knockout, M1/M21-DIKO) with a similar phenotype to the global Cyp27b1-KO mouse with low serum calcium and phosphate, low FGF23, very high levels of PTH as well as many skeletal and mineral defects (11). The M1/M21-DIKO mouse had minimal expression of the Cyp27b1 gene in the kidney and was resistant to endocrine hormone control by PTH, FGF23, or 1,25(OH)2D3. This animal also had altered
vitamin D metabolism with low circulating 1,25(OH)₂D₃ and elevated levels of 25(OH)D₃ among other metabolite changes (11). Critically, the M1/M21-DIKO mouse retained its basal expression and inflammatory induction of Cyp27b1 in non-renal tissues including macrophages and T-cells (11). We described this mouse as a kidney-specific, endocrine-deficient Cyp27b1 pseudo-null mouse since the effects were confined to the kidney (11). We also demonstrated that, like the global Cyp27b1-KO mouse, the M1/M21-DIKO mouse could have its skeleton and mineral defects rescued by feeding a diet high in calcium, phosphate, and lactose (2%, 1.25%, 20% lactose) for 12-16 weeks (11). This reduced the 1,25(OH)₂D₃ circulating levels below the lower limits of detection (LLOD) in the mouse. Therefore, we believe the dietary rescued M1/M21-DIKO mouse represents the ideal model with which to study vitamin D₃ supplementation and the potential to isolate and demonstrate non-renal production of 1,25(OH)₂D₃.

Routine and clinical measurements of both 25(OH)D₃ and 1,25(OH)₂D₃ in the serum or plasma are typically made with ELISA kits or a radioimmunoassay (RIA) assay (19), however these methods lack detailed analysis of the multitude of vitamin D metabolite derivatives and can often be confounded by them (20, 21). Methods of liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) greatly increases the ability to see these derivatives in the serum or plasma with absolute quantitation (22). However, detection of tissue levels of vitamin D metabolites is not trivial given the high lipid and cholesterol content of tissues. Further, detecting the potential for production of those metabolites in tissues, as we propose here, is also complicated by the circulating 1,25(OH)₂D₃ production from the kidney. Recently, methods were developed for the targeted acquisition of spatial identification and relative quantitation of vitamin D metabolites through advances in mass spectrometry imaging (23). Serum detection of vitamin D metabolites by LC-MS/MS involves a chemical derivatization (CD) step to improve the ionization efficiency of steroidal compounds (20, 22). By taking this a step further, the derivatization can be accomplished on tissue sections (on-tissue chemical derivatization, OTCD) to enable tissue detection of vitamin D metabolites. Additionally, spatial information within the tissue can be obtained when linked to MALDI followed by Mass Spectrometry (MS) detection (23). Thus far, this OTCD-MALDI-MSI
pipeline of analysis has been completed to identify both 25(OH)D₃ and 1,25(OH)₂D₃ in the mouse kidney of wildtype mice in proof-of-principle experiments (23).

In this current study, we combined this latest technology of MSI utilizing OTCD-MALDI-MSI to identify and quantify the tissue level production of 1,25(OH)₂D₃ and 25(OH)D₃. With our M1/M21-DIKO mouse, we have eliminated the circulating 1,25(OH)₂D₃ and focus on the detection of 1,25(OH)₂D₃ resident in the tissues themselves. Using this unique MSI technique, we demonstrate detection and spatial location of 1,25(OH)₂D₃ and 25(OH)D₃ in tissues outside of the kidney. We hypothesize that vitamin D₃ supplementation will increase 25(OH)D₃ circulation and tissue availability, as well as the production of 1,25(OH)₂D₃ in tissues outside of the kidney in the M1/M21-DIKO mouse. This, in turn, may prove beneficial for inflammatory disease amelioration and provide the missing link between cell and human studies. In these studies, we have demonstrated non-renal detection and production of 1,25(OH)₂D₃ in vivo, as well as gene expression consequences, in response to vitamin D₃ supplementation.

Results

Mass spectrometry imaging validation of vitamin D metabolites in wildtype and Cyp27b1-KO mice

We used a similar workflow and procedure as previously reported for mass spectrometry interrogation of targeted metabolite imaging and plasma metabolites from tissues (23), which is summarized in Fig. 1. The mice lacking mature, full-length CYP27B1 protein (Cyp27b1-KO) are known to have elevated levels of substrate 25(OH)D₃ compared to their wildtype (WT) littermates (10, 11). We confirmed these elevated plasma levels of 25(OH)D₃ and that 1,25(OH)₂D₃ is absent compared to WT mice, in this study. In Supplemental Figure 1A (Fig. S1A), mice (male and female) were examined by LC-MS/MS for both 25(OH)D₃ and 1,25(OH)₂D₃ concentrations in the plasma. WT mice had an average plasma 25(OH)D₃ concentration of 15.4 ± 1.1 ng/mL and a 1,25(OH)₂D₃ concentration of 23.7 ± 1.9 pg/mL (Fig. S1), consistent with our prior studies (10, 11). The Cyp27b1-KO (C27KO) mice had a 25(OH)D₃ concentration of 77.9 ± 2.8 ng/mL (Fig. S1) and 1,25(OH)₂D₃ that was found in the linear range but fell below the lower limit of quantitation (<LLOQ), however these values were above the lower limit of detection (LLOD), and
are therefore included and displayed in red. Plasma LLOQ for 25(OH)D3 is ≤ 2 ng/mL and 1,25(OH)2D3 ≤ 5 pg/mL. Both the 25(OH)D3 and 1,25(OH)2D3 Cyp27b1-KO vs. WT were statistically significant with p < 0.001 in each case by t-test analysis. Both male and female mice were included in the replicates, no differences were found and therefore the results are reported as mixed. These mice confirm our previously measured plasma 25(OH)D3 and 1,25(OH)2D3 concentrations for both the WT and Cyp27b1-KO mice (10, 11).

We then investigated tissue MALDI-MSI for both WT and Cyp27b1-KO animals. Both WT and Cyp27b1-KO animals were fed a chow diet (3.4 IU/g of vitamin D) until 8 w of age. Mice were then perfused with PBS and tissues were collected. Animal body weights, bone mineral density, serum Ca, P, PTH, and FGF23 are reported in Fig. S3. Importantly, the intratissue systemic contribution of blood was not detected in the tissues, indicating the perfusion was complete as detailed in the Methods. MALDI-MSI was performed for kidney, liver, spleen, and thymus, as seen in Fig. 2. Representative MSI images are shown in Fig. 2 panels A-D with 25(OH)D3 (top) and 1,25(OH)2D3 (bottom) of each panel. Relative quantification from the raw MSI data for each panel and biological replicate sample are displayed in Fig. 2F and 2G. Representative images from all biological triplicates and the full table of metabolites are shown in Fig. S2 and Fig. S1, respectively. The current resolution of MSI is based on the laser used for tissue ablation, which is near 35 µm in size, thus ablating ~3-5 cells at a time. As can be observed in Fig. 2A (upper), the WT kidney shows small amounts of 25(OH)D3 present indicated by the low ion relative intensity or abundance (lower on scale, lighter blue) compared to areas of high ion relative intensity and abundance (higher on scale, orange and bright yellow). The WT kidney 1,25(OH)2D3 (Fig. 2A, lower) shows a high relative intensity of the 1,25(OH)2D3 metabolite. In contrast to the WT metabolite levels of the 25(OH)D3, the Cyp27b1-KO mouse (C27KO) has high levels of 25(OH)D3. As expected, the Cyp27b1-KO mouse is incapable of making 1,25(OH)2D3 and therefore the MSI images show very low levels of ion intensity and metabolite detection. From an overlay of a schematic of the mouse kidney anatomy (Fig. 2E), we find that 1,25(OH)2D3 appears in both the cortex and medulla, with the inner medulla having a much higher relative ion intensity than the cortex or outer medulla. Quantification of these data as shown in Fig.
2F measure the 25(OH)D$_3$ of the WT kidney to be 34.1 ± 3.1 ng/g and the $Cyp27b1$-KO 98.5 ± 6.1 ng/g. 1,25(OH)$_2$D$_3$ quantification (Fig. 2G) of WT kidney is 66.4 ± 3.4 pg/g and $Cyp27b1$-KO as 3.8 ± 0.4 pg/g, however, the LLOQ for 1,25(OH)$_2$D$_3$ by MSI (or by LC-MS/MS) are 5 pg/g therefore, while these numbers were calculated, they appear as red in color in the quantification for below LLOQ. These data validate the method against the prior publication for MSI of vitamin D metabolites (23) and are in line with those measured for the plasma values (Table S1) and from our prior work (10, 11).

The liver is the source of 25(OH)D$_3$ production by the CYP2R1 enzyme and thus, is expected to have the highest tissue levels of 25(OH)D$_3$ in the mouse. In Fig. 2B, MSI confirms this expectation as the levels of 25(OH)D$_3$ are high in both the WT and $Cyp27b1$-KO mice. Quantification of these data in Fig. 2F show there is no statistical difference in the levels of 25(OH)D$_3$ between the WT and $Cyp27b1$-KO mice (202.5 ± 13.9 and 212.7 ± 11.6 ng/g, respectively, Fig. 2F). The levels of 1,25(OH)$_2$D$_3$ (Fig. 2G) in the liver are low with 2 of the 3 replicates falling below the LLOQ for both WT (4.4 ± 0.7 pg/g) and $Cyp27b1$-KO (4.5 ± 0.7 pg/g, Fig. 2G). Both spleen and thymus contain very little 25(OH)D$_3$ for either the WT or $Cyp27b1$-KO mice (spleen, 7.1 ± 0.5 and 7.0 ± 0.8 ng/g; thymus, 4.6 ± 0.9 and 17.3 ± 2.0 ng/g, Fig. 2F). While the $Cyp27b1$-KO appeared to have more 25(OH)D$_3$ in the thymus compared to the WT, this was not a statistically significant increase. The levels of 1,25(OH)$_2$D$_3$ in the spleen for the WT mouse was below the LLOD (< 2 pg/g) and the $Cyp27b1$-KO was at the LLOQ (Fig. 2G). Similarly, the 1,25(OH)$_2$D$_3$ levels in the thymus for either mouse were also at or below the LLOQ. Taken together, data from the WT and $Cyp27b1$-KO mice confirm our expectations and/or previous data (23) that the WT kidney should contain the highest levels of 1,25(OH)$_2$D$_3$, the liver should be a reservoir for 25(OH)D$_3$, $Cyp27b1$-KO mice should have elevated 25(OH)D$_3$ in the kidney, and the remaining tissues would have very little to no detectable 1,25(OH)$_2$D$_3$.

**Vitamin D metabolite profile of the M1/M21-DIKO mouse**

Next, we defined the tissue metabolite levels of our experimental M1/M21-DIKO mice. These mice have a genomic deletion in enhancers that render the $Cyp27b1$ gene incapable of responding to any
hormonal signals from FGF23, PTH, or 1,25(OH)₂D₃ as well as a reduced basal expression of \textit{Cyp27b1} (11). As mentioned, the important feature of the M1/M21-DIKO mouse is that any non-renal, inflammatory-induced response of \textit{Cyp27b1} is unchanged from WT expression. Therefore, we hypothesize that the M1/M21-DIKO mouse is an ideal model with which to test the non-renal production of 1,25(OH)₂D₃ in inflammation and inflammatory disease progression with the absence of renal produced 1,25(OH)₂D₃. Since the plasma levels of vitamin D metabolites mirror that of the \textit{Cyp27b1}-KO mouse, we expected the tissue levels of vitamin D metabolites in the M1/M21-DIKO mouse to also mirror the MSI of the \textit{Cyp27b1}-KO mouse. In Fig. 3, we assessed the qualitative MSI profile of the M1/M21-DIKO versus the \textit{Cyp27b1}-KO, with both mice on chow diet (3.4 IU/g vitamin D). Here, unlike Fig. 2, qualitative MS images were reconstructed using Fleximaging in contrast to the semi-quantitative evaluation performed using MSiReader software (as in Fig. 2). The intensity scale was based on total ion count using the rainbow color scale rather than the internal standard peak normalization (parula color scale) used for semi-quantitative assessment. Given this difference, these values are qualitative and not semi-quantitative (like Figs. 2 and 4). We found that in both the kidney and the liver (Fig. 3A and 3B), the M1/M21-DIKO (DIKO) mouse was comparable to the \textit{Cyp27b1}-KO (C27KO) mouse. The 25(OH)D₃ levels were high in the kidney tissue, and the 1,25(OH)₂D₃ levels were low in the M1/M21-DIKO mouse. We previously found that M1/M21-DIKO mice on chow did have low, but detectable levels of 1,25(OH)₂D₃ in the plasma compared to \textit{Cyp27b1}-KO (11). This appears to replicate in the kidney MSI with slightly elevated levels of 1,25(OH)₂D₃ as evidenced by increased ion intensity compared to the \textit{Cyp27b1}-KO, however the relative quantitation was not performed. The liver contained elevated levels of 25(OH)D₃ and no 1,25(OH)₂D₃ metabolites.

\textbf{Effect of vitamin D₃ supplementation on tissue metabolite levels in the M1/M21-DIKO mouse}

Since the M1/M21-DIKO mouse lacks kidney \textit{Cyp27b1} expression and regulation, we can exploit these features to associate vitamin D₃ supplementation of varying levels of vitamin D diets with health and disease outcomes. Therefore, we examined the tissue levels of both 25(OH)D₃ and 1,25(OH)₂D₃ after vitamin D₃ supplementation. We first need to rescue the mineral deficiencies of the M1/M21-DIKO mouse to recover
and maintain skeletal density and growth. Through dietary rescue with a high calcium, high phosphate, lactose containing diet, the M1/M21-DIKO mice grow to similar size and similar skeletal density to age matched WT mice (11). We found that this dietary rescue requires a minimum of 12 w of rescue diet (2% Ca, 1.25% P, 20% lactose) to recover serum Ca, P, and iFGF23 including lowering the serum PTH to WT levels (Fig. S3) and all values replicated our recent study (11). Therefore, to establish levels of vitamin D metabolites in the tissues, we first rescued mice for 12 w using the standard rescue diet (2% Ca, 1.25% P, 20% lactose containing 1.5 IU/g of vitamin D), and then switched to a diet containing either 0 IU/g of vitamin D (vitamin D deficient diet) or diet containing 20 IU/g of vitamin D daily (high D diet) for another 4 w resulting in a total experimental timeline of 16 w. We examined the extremes (0 IU and 20 IU) by MSI to capture a maximal potential effect (24). This high dietary supplementation at 20 IU was previously shown to elevate the serum levels and tissue levels of 25(OH)D$_3$, which was our desired outcome (24). We also examined a 5 IU vitamin D$_3$ supplementation as a moderate dose, however given technical constraints, these mice were not examined by MSI in this study and will be examined in a future study. The mice after the additional 4 w of 0 IU or 20 IU diet were unchanged outside of femoral BMD in the female mice (Fig. S3).

In Fig. 4A and B, we examined the plasma levels of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ by LC-MS/MS. The WT and Cyp27b1-KO mice (chow diet) were compared to the M1/M21-DIKO (DIKO, chow diet) mice as well as the M1/M21-DIKO mice with a 12-w rescue diet followed by 4 w either 0 or 20 IU vitamin D diet (DIKO-12wR-4w 0IU, DIKO-12wR-4w 20IU) with the full values included in Fig. S1. It is important to note that these mice were on different diets given the goals of this study: to remove circulating 1,25(OH)$_2$D$_3$ in the M1/M21-DIKO mice and test vitamin D$_3$ supplementation. Rescue diets and vitamin D$_3$ supplementation diets for WT and Cyp27b1-KO mice can cause different serum vitamin D metabolite profiles (24) and will be tested in the future. In Fig. 4A, we see the M1/M21-DIKO mice on the chow diet shows a similar (not significantly different) 25(OH)D$_3$ profile compared to the Cyp27b1-KO mouse (DIKO, 62.5 ± 4.2 ng/mL; C27KO, 77.9 ± 2.8 ng/mL, Fig. 4A). The 0 IU vitamin D diet did reduce the plasma 25(OH)D$_3$ concentration in the DIKO mice (62.5 ± 4.2 ng/mL to 36.0 ± 5.7 ng/mL, Fig. 4A) and the increase of vitamin D to 20 IU raised the 25(OH)D$_3$ concentration to 79.6 ± 5.1 ng/mL (Fig. 4A). The M1/M21-
DIKO mouse was on a normal chow diet (unrescued; mouse phenotype: high PTH, low Ca, P, and FGF23) for 8 weeks prior to the harvest for metabolites and these serum metabolites match our previous study (11). The M1/M21-DIKO mice were fed 12 w rescue followed by 4 w of 0 IU or 20 IU diet equaling 16 w of total diet post-wean. The 1,25(OH)₂D₃ levels in Fig. 4B show the normal WT levels of 23.7 ± 1.9 pg/mL are greatly reduced in the Cyp27b1-KO, M1/M21-DIKO, and the M1/M21-DIKO with the 0 IU diet, many of which are at or below the LLOQ but above the LLOD (red data points) (3.7 ± 0.2, 5.7 ± 0.3, and 6.0 ± 0.9 pg/mL, respectively, Fig. 4B). Surprisingly, the 1,25(OH)₂D₃ concentration in the M1/M21-DIKO mouse with the 20 IU diet was significantly increased to 17.8 ± 1.3 pg/mL (Fig. 4B), which was approaching WT levels (~ 23 pg/mL) though still significantly reduced (p < 0.05). These increased 1,25(OH)₂D₃ levels could be coming from an increased production in the kidney, or perhaps from non-renal tissues, therefore we turned to the MSI in the kidney, liver, spleen, and thymus to examine this possibility.

The MSI data for the kidney, liver, spleen, and thymus is shown in Fig. 4C and 4D with the quantification listed in Fig. 4G and 4H. A complete table of the quantification and remaining representative images are shown in Fig. S1 and S2. The first and most obvious location for the increased 1,25(OH)₂D₃ in the 20 IU diet could be from the kidney. As seen in Fig. 4C, reduction of vitamin D in the diet (0 IU) and reduction of vitamin D in the plasma (Fig. 4A), leads to a reduced amount of 25(OH)D₃ in the kidney tissue in comparison to the 20 IU diet as evidenced by the increased ion relative intensity. The quantification of the increase is detailed in Fig. 4G where the 25(OH)D₃ of the M1/M21-DIKO mouse with 0 IU diet is unchanged from the WT mice. However, the M1/M21-DIKO mouse with the 20 IU diet is significantly increased compared to the Cyp27b1-KO mouse (C27KO, 98.5 ± 6.1 ng/g; DIKO 20 IU, 174.0 ± 14.4 ng/g, Fig. 4G). Despite this increase in the 25(OH)D₃ levels, it did not appear to cause any increased 1,25(OH)₂D₃ production in the kidney of the M1/M21-DIKO mouse on the 20 IU diet. The MSI images (Fig. 4C, lower) show little to no presence of 1,25(OH)₂D₃ and the quantification in Fig. 4H demonstrates that the M1/M21-DIKO mice with either 0 IU or 20 IU diet are below the LLOQ yet above the LLOD, equivalent to the Cyp27b1-KO mice (C27KO, 3.8 ± 0.4 pg/g; DIKO 0 IU, 5.3 ± 0.6 pg/g; DIKO 20 IU, 5.6 ± 0.7 pg/g, Fig.
It would appear from these data that the source of the increased 1,25(OH)\textsubscript{2}D\textsubscript{3} in the plasma is not derived from the kidney.

The reservoir of 25(OH)D\textsubscript{3} in the liver of the WT and Cyp27b1-KO mice is reduced in the M1/M21-DIKO mice on the 0 IU diet (WT, 202.5 ± 13.9 ng/g; C27KO, 212.7 ± 11.6 ng/g; DIKO 0 IU, 95.3 ± 5.3 ng/g, Fig. 4G), and is markedly increased in the M1/M21-DIKO mice on the 20 IU diet (287.5 ± 13.7). The WT, Cyp27b1-KO, and M1/M21-DIKO 0 IU all have 1,25(OH)\textsubscript{2}D\textsubscript{3} levels at or below the LLOQ, however, the M1/M21-DIKO 20 IU diet shows an increase in 1,25(OH)\textsubscript{2}D\textsubscript{3} levels (18.4 ± 5.4 pg/g, Fig. 4H). While these biological replicates are variable, they were significantly increased and the MSI images for the 1,25(OH)\textsubscript{2}D\textsubscript{3} showed very punctate regions of higher ion intensity. The spleen and thymus both show lower levels of 25(OH)D\textsubscript{3} that are at or below the LLOQ and both tissues have an elevation of 25(OH)D\textsubscript{3} in the M1/M21-DIKO mice on the 20 IU diet. Unlike the modest elevations in the 25(OH)D\textsubscript{3}, the spleen had significant increases in 1,25(OH)\textsubscript{2}D\textsubscript{3} in both M1/M21-DIKO dietary samples (WT, <LLOD; C27KO, 5.4 ± 0.8 pg/g; DIKO 0 IU, 16.3 ± 2.3 pg/g; DIKO 20 IU, 57.8 ± 4.7 pg/g, Fig. 4H) and the MSI images depict a broad elevation of ion intensity of metabolite across the tissue. In Fig. 4I, we examined the spatial features of MSI. The splenic data (from Fig. 4E) was overlaid with an H&E staining of a section taken immediately following the MSI tissue section. We found that while 25(OH)D\textsubscript{3} was rather broadly increased, it appears that the 1,25(OH)\textsubscript{2}D\textsubscript{3} intensity increases in the white pulp segments of the thymus which are rich in T cells and macrophages that encompass the periarteriolar sheaths, follicles, and marginal zones (25). Finally, 1,25(OH)\textsubscript{2}D\textsubscript{3} in the thymus was also increased modestly with DIKO 20 IU diet (WT, 5.5 ± 0.9 pg/g; DIKO 20 IU, 16.9 ± 1.7 pg/g, Fig. 4H). These data indicate that these non-renal tissues, and possibly others not examined, are both 1) transporting 25(OH)\textsubscript{2}D\textsubscript{3} into these tissues and 2) may be leading to the elevation of 1,25(OH)\textsubscript{2}D\textsubscript{3} detected in the plasma by LC-MS/MS analysis (Fig. 4B).

To confirm the validity of the MSI relative quantitation data for each of these tissues, we conducted an important homogenate analysis of the remaining tissue block by LC-MS/MS absolute quantitation. We processed the Cyp27b1-KO (chow), M1/M21-DIKO (0 IU and 20 IU rescue diet) samples for this confirmation as described in the Methods. 25(OH)D\textsubscript{3} levels from the tissue homogenate mirrored those
from the MSI data analysis (Fig. 5A, example: C27KO – MSI, 98.5 ± 6.1 ng/g vs Tissue Homogenate, 95.3 ± 9.4 ng/g, remaining data found in Fig. S1). Similarly, data for 1,25(OH)2D3 (Fig. 5B) corroborate their MSI counterparts well thus confirming the validity of the MSI data. In further validation of the method, a Bland-Altman analysis was performed (examples shown in Fig. S4) (26). Despite the limited n values (n=3), this analysis showed an average bias of 1.013 ± 0.121 and the 95% Limits of Agreement ranged from 0.85 ± 0.06 to 1.21 ± 0.12 across each grouping of samples between the methods indicating that these methods are highly comparable.

**Gene expression consequences of elevated tissue 1,25(OH)2D3**

With elevated 1,25(OH)2D3 expression in tissues, we hypothesized that perhaps these tissues would have elevated levels of vitamin D target genes. We first confirmed the elevation of megalin and cubulin expression in the kidney versus non-renal tissues (Fig. S5). In Fig. S5, there is 100-fold more megalin (Lrp2) and cubulin (Cubn) found in the kidney, so 25(OH)D3 availability may be the highest. Furthermore, for these target genes to be responsive to 1,25(OH)2D3, the tissue should have expression of the vitamin D receptor (VDR). Therefore, we also evaluated the levels of Vdr expression in each of the tissues examined by MSI and in the M1/M21-DIKO mice that were fed either the 12-w rescue diet followed by 4 w of 0 IU or 20 IU diet. As can be seen in Fig. 6A, the kidney had the highest expression of Vdr, and this expression was increased with the feeding of the 20 IU diet. The liver was 100-fold lower expression than the kidney and the spleen and thymus were both 10-fold lower and unchanged in either diet condition. We next wanted to assess the downstream consequences of higher 1,25(OH)2D3 production in the tissues by looking at the target gene of Cyp24a1, one of the most sensitive vitamin D target genes. As can be seen in Fig. 6B, the kidney retained the highest level of Cyp24a1 expression that was increased by the 20 IU diet. The remaining tissues were 1000-fold lower in expression and were unchanged by diet. The expression of Cyp24a1 was at the threshold of detection by qPCR analysis in all tissues outside of the kidney in the absence of exogenous 1,25(OH)2D3 (9). It appeared that either the elevated plasma levels of 1,25(OH)2D3 in the 20 IU diet (Fig. 4B and S1) may be causing an elevation of Cyp24a1 and Vdr in the kidney, or the retention of
25(OH)D₃ in the kidney has reached levels high enough to activate the VDR directly to increase both *Cyp24a1* and *Vdr* (24, 27-29). The expression of *Cyp27b1* was unchanged in any diet condition (data not shown).

If the hypothesis of localized 1,25(OH)₂D₃ production affecting the inflammatory program of the immune cells is true, then we may see differential regulation of cytokines involved in pro- and anti-inflammatory responses (2, 30). In Fig. 6C, we examined the gene expression of several anti-inflammatory cytokines (*Il4*, *Il10*) and pro-inflammatory cytokines (*Tnfa*, *Il17b*, *Il6*, and *Il1b*) in the spleen, which was the tissue with the highest 1,25(OH)₂D₃ concentration demonstrated by both MSI and confirmatory tissue homogenate. 1,25(OH)₂D₃ has been shown to suppress the proinflammatory cytokines like *Tnfa* and *Il17b* and increase anti-inflammatory cytokines like *Il4* and *Il10* in macrophages and T cells during polarization, inflammatory disease, and cell differentiation (31-34). We found that *Il4* was significantly increased in the spleen, whereas *Tnfa* was suppressed. The remaining cytokines were not statistically changed. These data demonstrate a potential outcome of the production of 1,25(OH)₂D₃ in the spleen.

**Discussion**

The landmark finding of this study is the detection and measurement of non-renal vitamin D metabolite production in the absence of circulating 1,25(OH)₂D₃. This was only made possible by using our M1/M21-DIKO animal and its unique phenotype that mirrors the *Cyp27b1*-KO while retaining a mature CYP27B1 protein (all tissues) and non-renal inflammatory induction of *Cyp27b1* (11). A human study of anephric patients was recently published that corroborate our findings on the potential for non-renal 1,25(OH)₂D₃ production in the absence of kidney 1,25(OH)₂D₃ production (17). Our unique animal model gives us the flexibility to manipulate dietary conditions, model different diseases, and isolate tissues for direct measurements. Here, we isolated the ability of vitamin D₃ supplementation to elevate tissue distribution of both 25(OH)D₃ and 1,25(OH)₂D₃ in the animal and assess the downstream consequences after our dietary rescue. Using our targeted-metabolite MSI platform, we were able to identify and confirm tissue distribution of 25(OH)D₃ and 1,25(OH)₂D₃ in the wildtype mice and the control animal, the *Cyp27b1*-KO
mouse. Interestingly, the relative quantitation revealed that the WT mouse contained approximately 3-fold higher levels of 1,25(OH)₂D₃ in the tissue (66.4 ± 3.4 pg/g, Fig. 4H and S1) compared to 1,25(OH)₂D₃ measured in the plasma (23.7 ± 1.9 pg/mL, Fig. 4B and S1). The 25(OH)D₃ levels on the other hand were also elevated in the kidney tissue (34.1 ± 3.1 ng/g, Fig. 4A and S1) versus the plasma measurements (15.4 ± 1.1 ng/mL, Fig. 4G and S1). This provides an interesting observation and perhaps avenue to explore the export of 1,25(OH)₂D₃ from the tissue into the plasma, or it may be a consequence of the degradation of 1,25(OH)₂D₃ (or 25(OH)D₃) to 1,24,25(OH)₃D₃ (or 24,25(OH)₂D₃) prior to exit from the tissue. 1,24,25(OH)₂D₃ and 24,25(OH)₂D₃ were not measured in this study but are a focus for future experiments.

Outside of the kidney, we expected the liver to be a repository of 25(OH)D₃ and it was, with levels that were again quite elevated from the plasma (35, 36). The thymus and spleen had very little 25(OH)D₃ and levels of 1,25(OH)₂D₃ (Fig. 4) that were at or below the LLOQ or LLOD on a diet with normal vitamin D levels, which was our expectation. The Cyp27b1-KO mice were an excellent control and recapitulated the expectations from the plasma analysis of vitamin D metabolites. The Cyp27b1-KO mice are known to have elevated levels of 25(OH)D₃ and this was apparent in both in the liver and kidney tissues (10). This elevation is due to two phenomena: 1) inactivation of the CYP27B1 enzyme, thus no conversion, and more importantly, 2) secondary hyperparathyroidism that suppresses the activity and expression of the Cyp24a1 gene and CYP24A1 enzyme (9, 11). The levels of 1,25(OH)₂D₃ are 1000-fold less than 25(OH)D₃ in the plasma (picograms vs nanograms) and thus the driving force behind the substrate excess is likely more due to this suppression of Cyp24a1 and the degradation of 25(OH)D₃ to 24,25(OH)₂D₃. Our previous studies show that when PTH levels are high, there is very little conversion to 24,25(OH)₂D₃ (9-11). The MSI captures these features faithfully to previous observations as well as highlighting the fact that while plasma levels of 1,25(OH)₂D₃ are easiest to measure clinically, the tissue levels appear to be a significant variable and may vary widely in patient populations. Additionally, the ratios of 25(OH)D₃ to not only 24,25(OH)₂D₃ but also to the production of the 25(OH)D₃-26,23-lactone, a metabolite that is in less flux than 24,25(OH)₂D₃, are more valuable to determine output of vitamin D metabolism in clinical patients (37-39). These additional metabolites will be examined in future studies.
Based on our previous skeletal rescue in the M1/M21-DIKO mice (11), we chose to first establish a rescued animal in both the skeleton and PTH levels in the plasma prior to changing the vitamin D levels in the diet. We had previously optimized this diet for skeletal rescue over a minimum of 12 w (11). We then changed the diet for an additional 4 w with varying levels of vitamin D₃ supplementation, selecting the 0 IU diet and 20 IU diet as the extremes. We chose the 20 IU diet based on prior studies that demonstrated increased 25(OH)D₃ availability in tissues and high elevation of serum 25(OH)D₃ after 12 w of feeding (24) to ensure transport differences in the tissues, based on megalin/cubulin expression, were overcome to deliver 25(OH)D₃ into the non-renal tissues. At the 4-w time point, the mice on the 0 IU diet are not completely vitamin D “deficient” as evidenced by the plasma levels of 25(OH)D₃ measured (Fig. S1), however with extended feeding of this diet we have achieved plasma 25(OH)D₃ levels below the LLOD by LC-MS/MS absolute quantitation (data not shown). The 0 IU diet does achieve the stated purpose for our study at 4 w, as the MSI shows that tissue levels are greatly depleted of 25(OH)D₃ in the kidney (Fig. 3 - M1/M21-DIKO and Fig. 4 - 0 IU diet). Elevation of vitamin D₃ in the diet to 20 IU/g, increases the 25(OH)D₃ in the plasma, liver, and kidney. This elevation though, did not lead to any more 1,25(OH)₂D₃ being made in the kidney of the M1/M21-DIKO mouse. Importantly, now we find 25(OH)D₃ by MSI in the spleen and modestly in the thymus, providing the substrate for potential conversion to 1,25(OH)₂D₃. In turn, we found that the 1,25(OH)₂D₃ in the spleen was elevated to levels that rivaled the WT kidney tissue (spleen 20 IU, 57.8 ± 4.7 pg/g; kidney WT, 66.4 ± 3.4 pg/g, Fig. 4). It is possible then that the spleen is contributing to the elevated plasma levels of 1,25(OH)₂D₃ that are found in the M1/M21-DIKO mice on the 20 IU diet, however, there are many other tissues that may also be contributing in a similar manner that were not analyzed by MSI in this study such as the parathyroid, skin, or intestinal tissues.

We also found the elevation in liver 1,25(OH)₂D₃ an intriguing result (Fig. 4). The MSI shows punctate regions of increased ion intensity. The resolution of MSI does not yet allow cellular resolution but it is possible that there are small cell populations like stellate cells or infiltrated macrophages resident in the liver that are causing these punctate ion intensity signals (40, 41). As mentioned, the current resolution of MSI is near 35 µm in size, thus ablating ~3-5 cells at a time, so this method is incapable of individual cell
resolution. Similarly, the apparent low expression of Cyp27b1 in non-renal tissues could be adequate expression in a select subpopulation of cells surrounded by a high-abundance, homogenous mix of non-expressing cells. Other projects in the lab are focused on the discovery of these subpopulations. Finally, we are not proposing the 20 IU diet as a human clinical dietary intervention, it was chosen to portray a “high” or “maximal” effect in these studies to validate the MSI technique and dietary differences. Now that we have established this vital linkage between supplementation and non-renal production of calcitriol, we will examine different vitamin D3 supplementation levels in diets and their outcome not only in MSI, but also for inflammatory disease amelioration in M1/M21-DIKO, WT, and additional genotypes.

The power of MSI is in visualizing the spatial distribution of these vitamin D metabolites within the tissue themselves, in addition to the metabolite relative quantitation. We saw in Fig. 2A and from the schematic in Fig. 2E and extrapolating that to the Fig. 2A panels, that the 25(OH)D3 appeared to be focused in the kidney cortex and outer medulla, whereas 1,25(OH)2D3 appeared across the cortex, outer medulla, and inner medulla, with the highest intensity apparent at the inner medulla. We find it interesting that 1,25(OH)2D3 has a higher intensity in what appears to be a different location than peak 25(OH)D3 intensity. Our previous genomic data indicate there was an obvious tissue specific isolation of the Cyp27b1 enhancers to the proximal convoluted tubule cells (8). While these data are intriguing, we do not understand these distributions and why it appears the locations differ between the substrate and product. It is possible that some of this can be explained by the inevitable diffusion of metabolites during the OTCD processing and thus a technical aberration (23). It also highlights the need for continued development and experimentation in this powerful platform. Despite this, the MSI technique is clearly a methodology that can be used to study the transport of these vitamin D metabolites from tissues. For the spleen in Fig. 4I, we found that the 25(OH)D3 is widely distributed, but the 1,25(OH)2D3 appears to have higher relative ion intensity in what appear to be the germinal centers and perhaps the marginal zones within the white pulp of the spleen (42). These areas contain higher concentrations of B cells under development and macrophages. Macrophages are one cell type that has been known to generate 1,25(OH)2D3 in an inflammatory disease state (12, 43, 44). We are now focused on refining this resolution and limiting diffusion to focus on specific structures,
cell populations, and regions of interest. One such application will be in defining the contributions of vitamin D metabolites in immune cells or others at the point of disease contact in areas such as colonic or atherosclerotic lesions. While 25(OH)D₃ and 1,25(OH)₂D₃ are important to identify, we are also focused on expanding this technique to capture the catabolic derivatives of these metabolites in 24,25(OH)D₃ and 1,24,25(OH)D₃ and others. We, and others, have demonstrated relationships between metabolites such as 25(OH)D₃ and 24,25(OH)D₃ that can be predictive on PTH levels and skeletal health (37, 45, 46). As we expand our studies to disease models, we will explore the ratios of 25(OH)D₃:24,25(OH)₂D₃ and 25(OH)D₃:25(OH)D₃-26,23-lactone in tissues compared to the plasma.

In the final set of experiments, we looked at the impact of 1,25(OH)₂D₃ production in the tissues on gene expression. For 1,25(OH)₂D₃ to have a consequence, the VDR should be expressed in these tissues. We confirmed previous studies (10, 11) for the concentrations of Vdr expressed in the tissues we isolated in that kidney was the highest, followed by thymus and spleen, with the liver having very low levels of Vdr detected by qPCR (Fig. 6). We also confirmed that Cyp24a1 expression was very low in these non-renal tissues, often at the threshold of detection by qPCR analysis. Cyp24a1 was not elevated in these tissues after high vitamin D₃ supplementation facing physiologic levels of 1,25(OH)₂D₃. We have found that supraphysiologic injections of 1,25(OH)₂D₃ easily activate the Cyp24a1 gene in any tissue that contains the VDR (10, 11). This is different than the activities in the kidney post 20 IU vitamin D₃ supplementation. Here in the kidney, the physiologic elevations in 1,25(OH)₂D₃ lead to activation of Cyp24a1. We believe that this indicates two different modes for Cyp24a1 activity in the body: 1) renal regulation of Cyp24a1 responds to and maintains the circulating blood 1,25(OH)₂D₃ levels, and 2) non-renal Cyp24a1 exists for emergency detoxifying circumstances. When faced with physiologic levels of 1,25(OH)₂D₃, those non-renal tissues preserve 1,25(OH)₂D₃ for positive gene expression (no Cyp24a1 expressed), when exposed to higher levels of 1,25(OH)₂D₃, the detoxification program is initiated in all tissues. If vitamin D is anti-inflammatory as the literature would suggest, we should see consequences of these 1,25(OH)₂D₃ changes in immune cells and in tissues that harbor immune cells like the spleen (2, 31, 34, 47). We did, in fact, see a change in both Tnfa and Il4 in the spleen. These cytokines were unchanged in the liver and thymus (data
In prior studies with Cyp27b1-KO mice, it was found that the 20 IU vitamin D3 dietary supplementation fed for 12 weeks was able to induce gene expression in the intestine (24). While we used a shorter term for vitamin D3 supplementation (4 w), and we found very little 25(OH)D3 in tissues outside of the kidney and liver, it is still possible these elevated levels of 25(OH)D3 are causing gene regulation in the spleen. 25(OH)2D3-activated VDR is very difficult to control for experimentally in the animal, therefore, we will continue to examine these diets at varying vitamin D3 concentrations to help clarify 25(OH)D3-activated VDR expression. Given our gene expression results in the spleen, it appears this elevation of splenic 1,25(OH)2D3 may affect the immune program. We are currently focused on isolation of different immune populations for closer examination of their expression and differentiation under these dietary conditions. We are also examining these cell populations in inflammatory disease models for their contribution to amelioration of disease.

In conclusion, this work is the first example of leveraging the power of the OTCD-MALDI-MSI platform to capture and quantify the renal and non-renal production of vitamin D metabolites. We can manipulate the plasma levels of 25(OH)D3 through vitamin D3 supplementation, not 1,25(OH)2D3 or 25(OH)D3 dosing, and this appears to have consequences to gene expression and production of 1,25(OH)2D3. Continued development of this platform will enable us to identify the connection between plasma vitamin D levels, or status, and disease benefits that we believe will help guide future clinical recommendations.

**Methods**

*Sex as a biological variable:* For all studies, both male and female mice were included in the analyses. Sex was considered as a biological variable and data were reported as mixed where no differences were found in the data.
**Materials:** 1α,25(OH)2D3, acetone, hexane, zinc sulphate, gelatine, 2-mercaptobenzotiazole (MBT) and sodium borohydrde we purchased from Sigma-Aldrich (UK). 25(OH)D3, commercially available lyophilized four-point calibration standards were purchased from (Chromsystem, München, GmBH). Amplifex® Dieen reagent was purchased from Sciex (Chemistry and Consumables R&D, Framingham, MA, US). Isotopically labelled d6-25(OH)D3 and d6-1α,25(OH)2D3 were purchased from Cambridge Isotope Laboratories (MA, US). 25(OH)D3 serum calibrators were purchased from Chromsystem GmbH. LC-MS grade acetonitrile (ACN), methanol and formic acid were obtained from Fisher (UK). The water used was generated through a direct-Q Ultrapure water system from Millipore (Bedford, MA) with a specific resistance of 18.2 MΩ cm. Traditional genotyping PCR was completed with GoTaq (Promega, Madison, WI) and all real-time qPCR was completed with the StepOnePlus using TaqMan for gene expression assays (Applied Biosystems, Foster City, CA). Primers were obtained from IDT (Coralville, IA).

**Animal Studies:** All mice were derived from or created in the C57BL/6J genetic background as previously described (10, 11). Cyp27b1-KO, M1/M21-DIKO, and littermate wildtype (WT) mice aged 8-9 w (The Jackson Laboratory, Bar Harbor, ME) were housed in high density ventilated caging in the Biochemistry Animal Research Facility of the University of Wisconsin-Madison under 12-hour light/dark cycles at 72°F and 45% humidity. Mice used in this study were maintained on a standard rodent chow diet (5008, Lab Diet, St. Louis, MO), rescue diet (1.5 IU vit D/g, 2% Ca, 1.25% P, 20% lactose, TD.96348, Inotiv/Envigo, Madison, WI), 0 IU diet (0 IU vit D/g, 2% Ca, 1.25% P, 20% lactose, TD.200097, Inotiv/Envigo, Madison, WI), or 20 IU diet (20 IU vit D/g, 2% Ca, 1.25% P, 20% lactose, TD.200096, Inotiv/Envigo, Madison, WI). M1/M21-DIKO mice were fed the rescue diet for 12 w post-wean, followed by a diet switch to either the 0 IU or 20 IU diet for an additional 4 w (16 w total diet, mouse aged 19 w). All animal studies were reviewed and approved by the Research Animal Care and Use Committee of University of Wisconsin-Madison under protocol A005478. Unless otherwise indicated, all experiments were conducted with equal numbers of males and females (n ≥ 6). From these mice, a subset was chosen for MSI (n = 3) for each diet and genotype.
condition, including both M and F in the groups. MSI is a technically complex method which is not easily scaled to high n value experiments. We determined that n=3 provided statistically sound data for this study. Data were reported as mixed, as no differences were found between the sexes.

Tissue perfusion and collection: Mice were anesthetized under ether, opened, and blood was collected by cardiac puncture and split into serum or EDTA-treated plasma. Post puncture 10 mL of 1x PBS was perfused to remove blood from the circulation and tissues. Intratissue systemic contribution of blood in all target tissues was assessed by monitoring the heme b ion from myoglobin containing an iron ion in the +3 oxidation state as (Fe(III) + porphyrin (C_{34}H_{32}N_{4}O_{4}Fe)) at m/z 616.1772 ± 0.025 Da. No systemic contribution was observed for all tissues as signal/noise (S/N) of the heme b ion was below 3. For MSI, tissues were dissected, weighed, and drop frozen in liquid N₂ in 2.0 mL cryo vials. For gene expression, tissues were dissected and drop frozen in liquid N₂ in microfuge tubes. All tissues were stored long term in -80°C.

Blood Chemistry: Cardiac blood was collected at the time of sacrifice. Collected blood was split into serum or EDTA-treated plasma, incubated at room temperature for 30 min followed by centrifugation at 6,000 rpm for 12 min (x2) to obtain serum or EDTA-plasma (10). Serum calcium and phosphate levels were measured using QuantiChrom™ Calcium Assay Kit (#DICA-500, BioAssay Systems, Hayward, CA) and QuantiChrom™ Phosphate Assay Kit (#DIPI-500, BioAssay Systems, Hayward, CA) (10). Circulating intact FGF23 and PTH were measured in EDTA plasma via a Mouse/Rat FGF-23 (Intact) ELISA Kit (#60-6800, Immutopics, San Clemente, CA) and a Mouse PTH (1-84) ELISA kit (#60-2305, Immutopics), respectively (10).

Bone Mineral Density (BMD): BMDs were measured and analyzed by dual x-ray absorptiometry (DEXA) with a PIxImus2 densitometer (GE-Lunar Corp, Madison, WI) as previously described (48).
**Gene Expression:** Frozen tissues were homogenized in Trizol Reagent (Life Technologies) and RNA was isolated as per the manufacturer's instructions. 1 µg of isolated total RNA was DNase treated, reverse transcribed using the High-Capacity cDNA Kit (Applied Biosystems), and then diluted to 100 µL with RNase/DNase free water. qPCR was performed using primers specific to a select set of differentially expressed genes by TaqMan analyses. TaqMan Gene Expression probes (Applied Biosystems) used in Supplemental Table 1.

**Tissue Sectioning and Mounting:** Tissue preparation Sectioning was performed on frozen target tissues from each mouse using a Leica cryostat (CM 1850 UV; Leica Biosystems, Nußloch, Germany) with gelatine (10% v/v) as a mounting medium. Adjacent cross Sects. (12 µm thickness) were taken from a top-down (horizontal) plane and thaw-mounted onto conductive indium tin oxide (ITO)–coated slides (Bruker Daltonik, Bremen, GmbH & CO. KG). Further adjacent Sects. (10 µm thickness) were mounted onto a slide pre-coated with poly-l-lysine for histological staining. The remaining tissue was used for tissue homogenate analysis by LC-MS/MS. All sections were dried in a vacuum desiccator at room temperature for 30 min and stored at −80 °C for analysis.

**Instrumentation:** MSI experiments were performed on an Orbitrap® LTX-XL Mass Spectrometer (Thermo Fisher Scientific, GmbH, Bremen Germany) couple to a reduced pressure ESI/MALDI interface (Spectroglyph LLC, Kennewick, US). Confirmatory Liquid Chromatography-Tandem Mass Spectrometry analysis was performed using a triple-quadrupole linear Ion trap Mass Spectrometer SCIEX 5500+ Qtrap (Sciex, Framingham, US) couple with an Ultra high pressure Liquid Chromatography system Nexera® UPLC (Shimadzu, Kyoto, Japan).

**On-Tissue Chemical Derivatization (OTCD):** At -80 °C, tissue sections were dried in a vacuum desiccator (20 min). Amplifex reagent (5 mL, 0.1 mg/mL in 50:50 v/v acetonitrile/water) was applied to the tissue sections by protocol adapted from (23) using an artistic airbrush (AirFlow, SZ, China) instead of an
automated Image Prep due to restricted quantities of Ampiflex® reagent. Briefly, a reagent solution of Ampliflex (2 mL, 0.25 mg/mL in 50:50 v/v acetonitrile/water) with the incorporation of 10µL of ITSD (d6-25(OH)D3 at 1ng/ml was sprayed positioned 20 cm away from the target with a N₂ pressure of 1.2 bar. 2 ml/slide with each manual pass of approx. 1 s and repeated with 5-10 s between passes until uniform coating was achieved (9 of 0.015 mg/cm²). Then, the slide was placed in a closed reaction container, containing a 1:1 v/v solution of acetonitrile/water and a moist Kimwipe under the lid and placed in an oven at 37 °C for 1 h for derivatization completion. Sections were then dried under vacuum desiccator (25min) and ready for MALDI matrix application.

**Matrix Application:** Matrix Application 2-mercaptobenzothiazole (10 mL, 6 mg/mL in 90:10 v/v acetonitrile/water) was applied in eight passes using a 3D printer, as previously described(23, 49). A flow rate of 0.1 mL/min with a gas pressure of 2 bar, a bed temperature of 30 °C, a z-height of 30 mm, and a velocity of 1100 mm/min were achieved, averaging a run time of 30 min per slide. A uniform coating of matrix was achieved with 0.12 mg/cm².

**Mass Spectrometry Imaging Acquisition:** MSI experiments were performed on an Orbitrap LTQ-XL Mass Spectrometer (Thermo Fisher Scientific, GmbH, Bremen, Germany) couple with a reduced pressure Electrospray (ESI)/MALDI interface (Spectroglyph, Kennewick, WA, US). Further details on the interface can be found in Belov et al., 2017(50). The 349nm MALDI laser (Spectra Physics, MV, CA, US) was operated at a repetition rate of 1000Hz and pulse energy of ~2.3µJ. The Mass Spectrometer was operated in positive ion mode using a maximum ion injection time of 250ms in FT mode, automatic gain control (ACG) turned off and a mass range of 500-800Da. The spectral resolution was set to 60.000FWHM at m/z 400. The experiments were performed at 100x100 µm spatial resolution for liver and thymus tissue sections and 75x75 µm for kidney and spleen tissue sections. Optical images were taken using a flatbed scanner (Cannon LiDE-20, Cannon, UK). The instrument was calibrated using ESI Cal mix solution as per manufacture specifications. Collision induces dissociation experiments (CID) on both VitD metabolites
(1α,25(OH)2D3 and 25(OH)D3 Ampiflex derivatives was performed for structural confirmatory analysis at corresponding precursor masses m/z 732.50 and m/z 748.50 with an isolation window of 2 Da summing 10 scans/spectra using 16 and 17 V as collision energies (CE) respectively covering a scan range of 280-750Da.

**Spectra Processing:** Thermo Fisher raw data were aligned to the corresponding position files in Image Insight software (Spectroglyph, Kennewick, WA, US) and exported form there as ImzML files using the supplier recommended default parameters. The ImzML files were uploaded onto the open-source MSiReader version 1.02 9 (Raleigh, NC, US) using MATLAB version 2022b and Fleximaging version 5.1 (Bruker Daltonik, GmBH). All data was normalized using the d6-25(OH)D3 mass at (M+) 738.5435±0.025Da (peak normalization). MS images were generated for both vitamin D metabolites detected as Ampiflex derivatives at m/z 732.5058±0.025 for 25(OH)D3 and m/z 748.5008±0.025 for 1α,25(OH)2D3. For relative semi-quantitation (tissue mimetic model), intensities values for each metabolite as Ampiflex® derivatives were collected and ratios calculations against the ISTD (d6-25(OH)D3 were calculated. Relative quantitation was accomplished by plotting ratio intensities against matrix-matched standard calibration using the mimetic model. MSiQuant (part of the MSiReader software package) was used as software for calculations.

**Confirmatory LC-MS/MS analysis (absolute quantitation):** Please see supplementary methods.

**Histological Staining:** Please see supplementary methods.

**Statistical Analysis:** Data were analyzed using GraphPad Prism 10.2.2 software (GraphPad Software, Inc., La Jolla, CA) and in consultation with the University of Wisconsin Statistics Department. All values are reported as the mean ± standard error (SEM) and differences between group means were evaluated using One-Way ANOVA or Student’s t-test (1-tailed) as indicated in the figure legends. Bland-Altman was
performed in Graphpad Prism. Significance is determined by p-values with p < 0.05 and p values are reported in each figure and figure legend (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

**Study approval:** All animal studies were reviewed and approved by the Research Animal Care and Use Committee of University of Wisconsin-Madison under protocol A005478.

**Data availability:** Values for all data points and graphs for this study are reported in the Supporting Data Values file and are included in the supplemental materials. All images used and replicates are available in the supplemental materials. Data from MSI studies were processed using MSiQuant software from the MSiReader Software Package and compiled in Excel and GraphPad Prism.


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**FOOTNOTES**

Abbreviations used: 1,25(OH)2D3, 1,25 dihydroxy vitamin D; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; Ca, calcium; P, phosphate; M1, Mettl1 intronic enhancer of Cyp27b1; M21, Mettl21b intronic enhancer of Cyp27b1; M1/M21-DIKO, M1 and M21 double intronic enhancer knockout
out; Mass Spectrometry Imaging (MSI); LC-MS/MS, high pressure liquid chromatography – tandem mass spectrometry; On-tissue chemical derivatization (OTCD). Matrix Assisted Laser Desorption Ionization (MALDI)

References


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**Figure 1:** Mass spectrometry imaging workflow overview of tissue analysis. Isolated tissues are sectioned, and tissue samples are split for H&E staining, tissue homogenization (for absolute quantitation) and OTCD-MALDI-MSI for spatial distribution assessment and relative quantitation. Samples for OTCD-MALDI-MSI analysis undergo reagent application (Ampifix®) with the inclusion of a stable isotope for relative quantitation and subsequent MALDI matrix application for MALDI-MSI analysis. Protonated ions for targeted metabolites are selected for image generation and relative quantitation with further ion isolation for structural confirmatory analysis (MS/MS) The plasma is taken for systemic metabolite contribution and analyzed by HPLC-MS/MS along with tissue homogenate for absolute quantitation of vitamin D metabolites.
Figure 2: Tissue localization and relative quantitation of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ in Cyp27b1-KO and WT mice. MALDI-MSI was performed on both Cyp27b1-KO and WT littermates in the kidney (A), liver (B), spleen (C), and thymus (D). Scale for each metabolite shown to the right of each tissue image (additional images in supplemental data). Intensity was normalized by stable isotope internal standard protonated mass. Signal intensity is depicted by parula color scale for semi-quantitative assessment on the scale shown. E, schematic of kidney anatomy overlaid with panel from 1,25D WT from (A). The relative quantitation of biological triplicates is shown for the 25(OH)D$_3$ (F, ng/g) and 1,25(OH)$_2$D$_3$ (G, pg/g) for each tissue (all values in Fig. S1). Data points that fall below the lower limits of quantitation (<LLOQ) but above the lower limits of detection (LLOD) are shown in red. n=3 for all tissues. Unpaired t-tests were performed: ****, p<0.0001 C27KO vs WT.
Figure 3: Qualitative Comparison of Cyp27b1-KO and M1/M21-DIKO mice by MALDI-MSI. MALDI-MSI was performed and the representative images from the kidney (A) and liver (B) for both the Cyp27b1-KO (C27KO) and M1/M21-DIKO (DIKO) mice and 25(OH)D$_3$ (25D) and 1,25(OH)$_2$D$_3$ (1,25D) were measured and displayed. The intensity scale used was based on total ion count (TIC) normalization using rainbow color scale.
Figure 4: Detection of non-renal 1,25(OH)2D3 production after vitamin D3 supplementation. Plasma levels of 25(OH)D3 (A, 25D, ng/mL) and 1,25(OH)2D3 (B, 1,25D, pg/mL) as detected by LC-MS/MS in the wildtype littermates (WT), Cyp27b1-KO (C27KO), M1/M21-DIKO (DIKO), M1/M21-DIKO 12 w rescue diet followed by 4 w of 0 IU vitamin D diet (12wR-4w 0 IU), and M1/M21-DIKO 12 w rescue diet followed by 4 w of 20 IU vitamin D diet (12wR-4w 20 IU). One-way ANOVA with multiple comparison Tukey post-test: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, as indicated. MSI was performed and displayed for kidney (C), liver (D), spleen (E), and thymus (F) in the M1/M21-DIKO 12 w rescue diet followed by 4 w of 0 IU vitamin D diet (12wR-4w 0 IU) and M1/M21-DIKO 12 w rescue diet followed by 4 w of 20 IU vitamin D diet (12wR-4w 20 IU). Signal intensity is depicted by parula color scale for semi-quantitative assessment on the scale shown (additional images in supplemental data). The relative quantitation of biological triplicates is shown for the 25(OH)D3 (G, ng/g) and 1,25(OH)2D3 (H, pg/g) for each tissue (all values in Fig. S1). Data points that fall below the lower limits of quantitation (<LLOQ) but above the LLOD are shown in red. n=3 for all tissues. One-way ANOVA (genotypes within tissues) with multiple comparison Tukey post-test: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, as indicated. I, hematoxylin and eosin staining of thymus section with red and white pulp indicated. Overlaid data from spleen 25D and 1,25D panels from (E).
Figure 5: Confirmatory vitamin D metabolite levels in tissue homogenate. LC-MS/MS was performed on the remaining tissue not used for sectioning for 25(OH)D$_3$ (A, 25D, ng/g) and 1,25(OH)$_2$D$_3$ (B, 1,25D, pg/g) in the Cyp27b1-KO (C27KO), M1/M21-DIKO 12 w rescue diet followed by 4 w of 0 IU vitamin D diet (12wR-4w 0 IU), and M1/M21-DIKO 12 w rescue diet followed by 4 w of 20 IU vitamin D diet (12wR-4w 20 IU). Absolute quantitation of biological triplicates is shown for 25(OH)D$_3$ (A, ng/g) and 1,25(OH)$_2$D$_3$ (B, pg/g) for each tissue compared to the MSI values from Fig. 4 (all values in Fig. S1). Data points that fall below the lower limits of quantitation (<LLOQ) but above the LLOD are shown in red. n=3 for all tissues. One-way ANOVA (genotypes within tissues) with multiple comparison Tukey post-test: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, as indicated. There were no differences between groups in MSI vs. Tissue Homogenate (by ANOVA and t-test).
Figure 6: Gene expression changes in response to tissue elevations of 1,25(OH)₂D₃. Gene expression was performed in the kidney, liver, spleen, and thymus for mice on the M1/M21-DIKO 12 w rescue diet followed by 4 w of 0 IU vitamin D diet (12wR-4w 0 IU, n ≥ 14), and M1/M21-DIKO 12 w rescue diet followed by 4 w of 20 IU vitamin D diet (12wR-4w 20 IU, n ≥ 6) for Vdr (A) and Cyp24a1 (B). Cytokine gene expression was measured in the spleen for Il4, Il10, Il6, Tnfa, Il1b, Il2, and Il17a in the same diet conditions as A and B. Unpaired t-tests were performed: ****, p<0.0001; ***, p<0.001; *, p<0.05 - 20 IU vs 0 IU.