Non-Toxic Local Immunoregulation Using 1,25(OH)2 Vitamin D3 Prolongs Vascularized Composite Allograft Survival

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University of Pittsburgh | Department of Plastic Surgery

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Vascularized Composite Allotransplantation

- Reconstructive transplantation, involves the transfer of a limb or body feature **from one person to another.**
- Have become a clinical reality and valid treatment options for patients suffering from complex tissue injuries or defects.

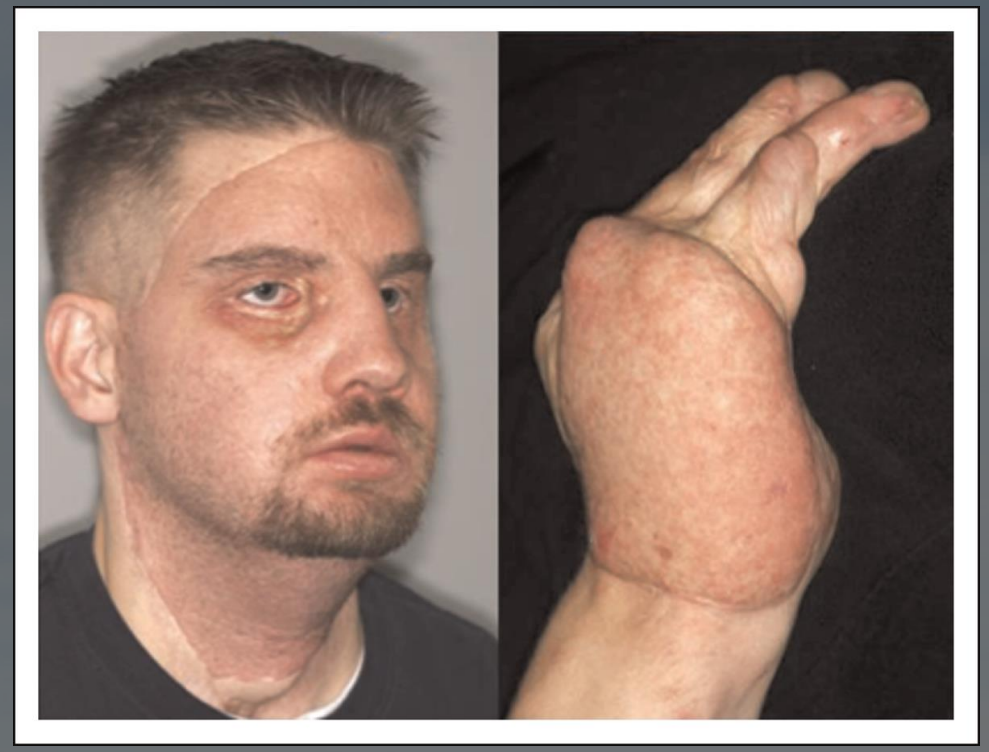
Source: Sosin M, et al. Plast Reconstr Surg. 2016;138(1):205–219.

Acute rejection remains a major challenge in VCA

- With reported rates **approaching 89%,** far exceeding those observed in solid organ transplantation

(AR is most common in lung transplantation = 29%)

- Potential cause: Skin is the most immunogenic component in VCA



Acute Rejection in VCA Face Transplant

Source: Fischer S, et al. Curr Opin Organ Transplant. 2014;19(6):531–544.

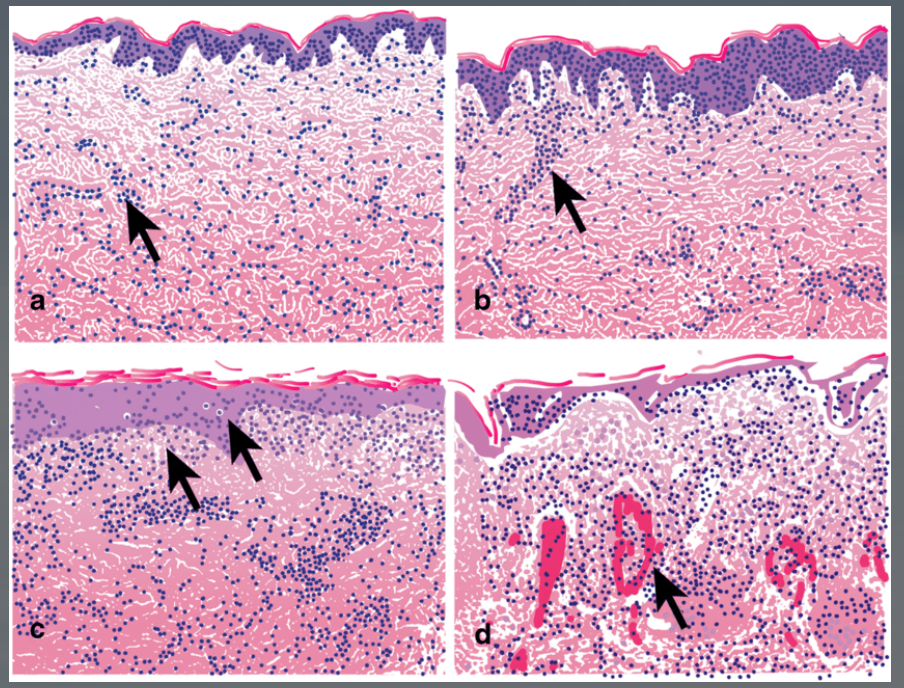
Acute Rejection in VCA

Immunosuppression protocols in VCA still relies on systemic solid organ transplant protocols

Treatment	Complications
Tacrolimus (FK506) Mycophenolate mofetil (MMF) Corticosteroids	Nephrotoxicity, Opportunistic infections Malignancy risk Poor wound healing

Acute Rejection in VCA

- Gold standard diagnosis: Skin biopsy + Banff criteria histopathology (invasive, disfiguring, traumatizes the allograft, requires expert interpretation).
- Treatment: Steroid pulse → tacrolimus/MMF escalation → salvage (ATG, plasmapheresis). ~20% of acute rejection episodes fail standard therapy.
- Overt clinicopathological changes occur late in the rejection process, making reversal difficult. No validated biomarkers exist to predict rejection before clinical manifestation.

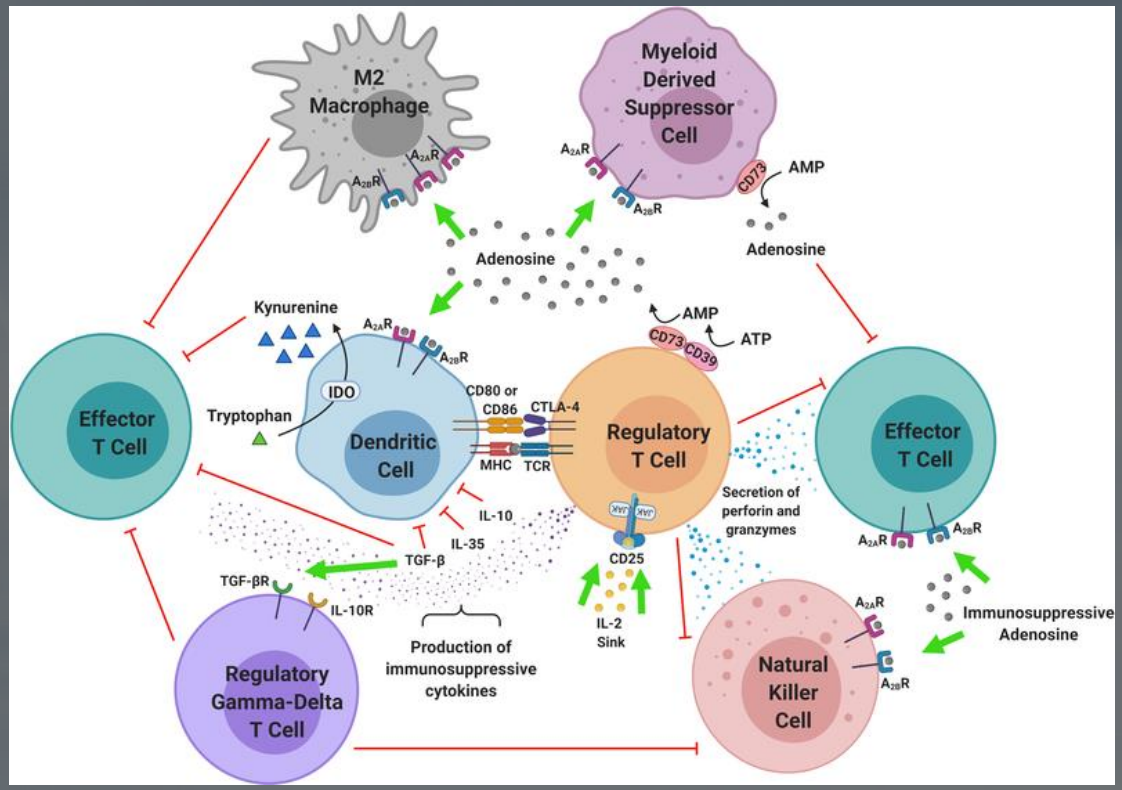


Banff classification of graft rejection

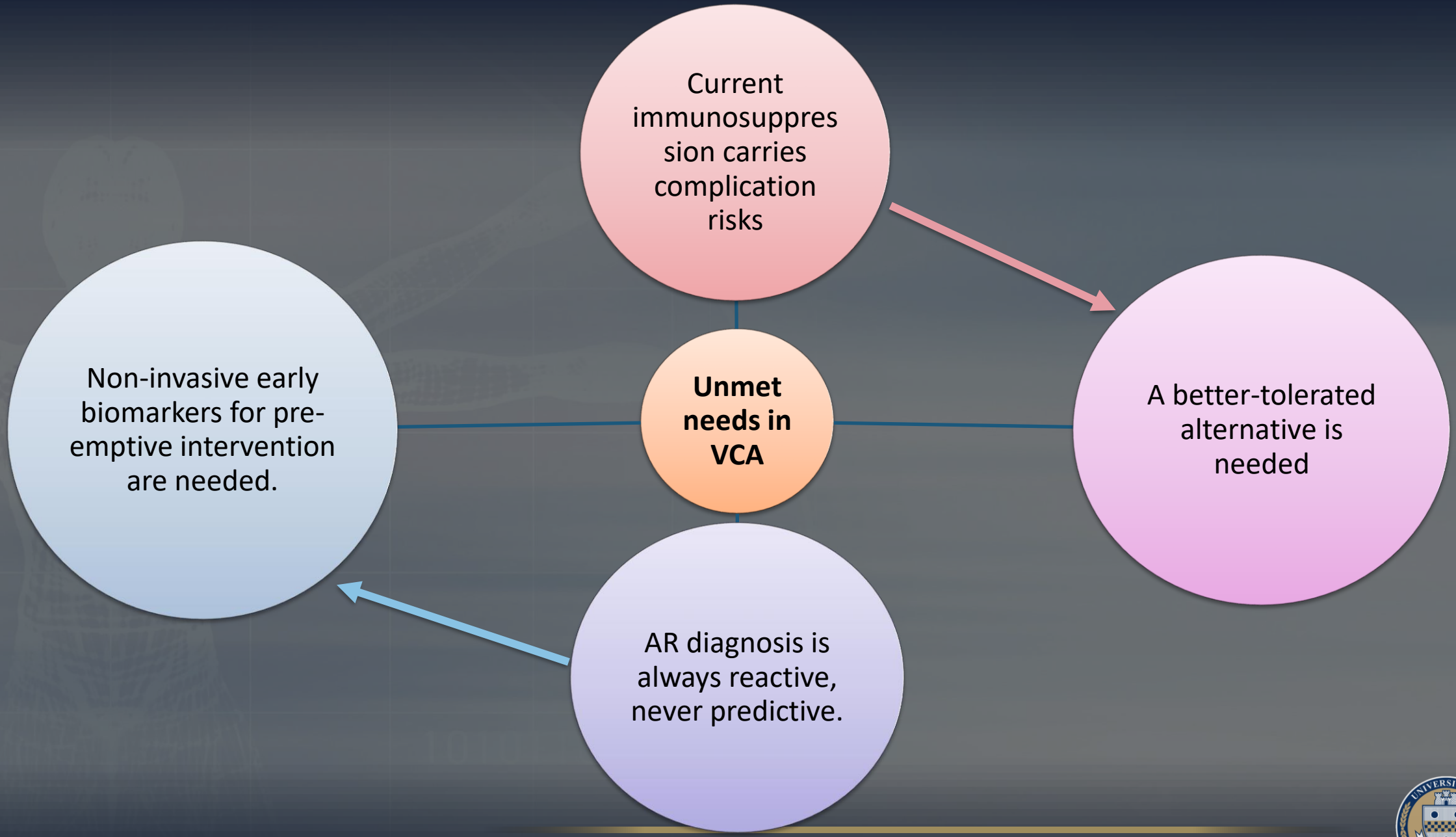
Source: Leonard D, et al. Curr Transplant Rep. 2020;7:1–9.

Prevention of Acute Rejection

- Local immune modulation:
  - Less toxic to life saving organs (e.g kidneys)
  - Stronger local immunological effects to the skin (highly immunological organ)



Dees, Sundee & Ganesan, Rajkumar & Singh, Sanjaya & Grewal, Iqbal. (2020). Regulatory T cell targeting in cancer: Emerging strategies in immunotherapy. European Journal of Immunology. 51. 280-291. 10.1002/eji.202048992.


Current immunosuppression carries complication risks
Non-invasive early biomarkers for pre-emptive intervention are needed.
Unmet needs in VCA
A better-tolerated alternative is needed
AR diagnosis is always reactive, never predictive.

Calcitriol: beyond a hormone

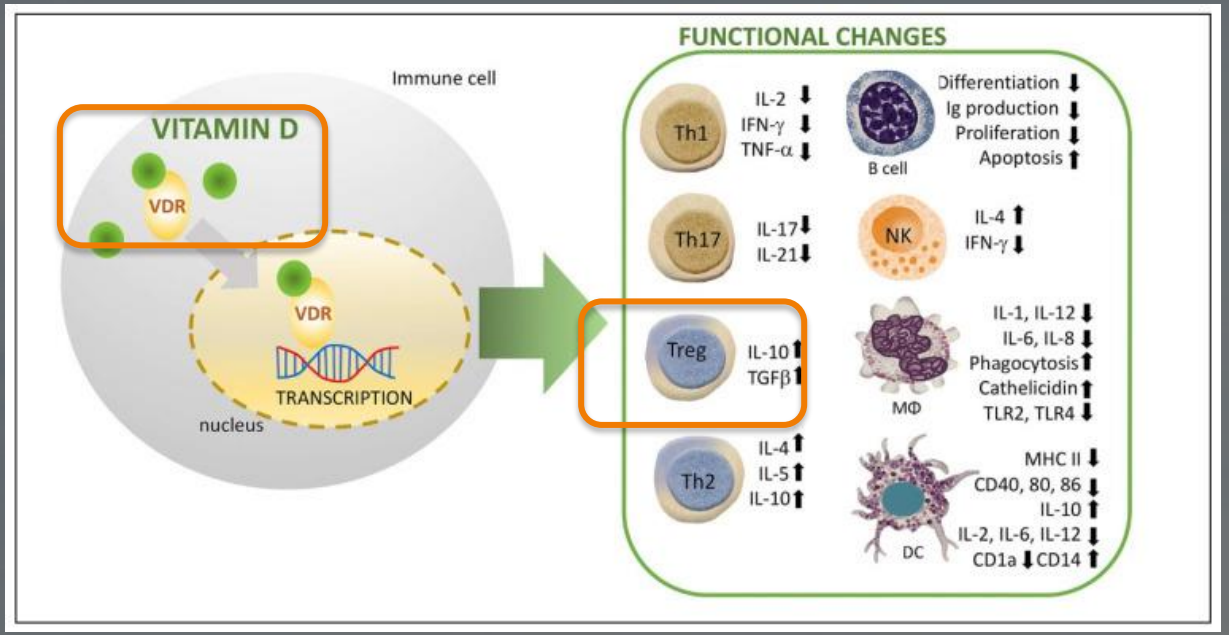
Calcitriol ($1,25(OH)_2D_3$) is the active form of Vitamin D, responsible for Ca and P mechanism

- Binds intracellular VDR → heterodimerizes with RXR → binds VDREs → regulates immune gene transcription

Treg induction:

Direct: VDR binds VDRE on *Foxp3* promoter + upregulates Helios (*Ikzf2*) → stable CD4+CD25+FoxP3+ Tregs → ↑IL-10, ↑TGF-β

Indirect: Calcitriol → tolerogenic DC → ↓MHC II, ↓CD80/CD86, ↑IL-10 → de novo peripheral Treg + Tr1 expansion



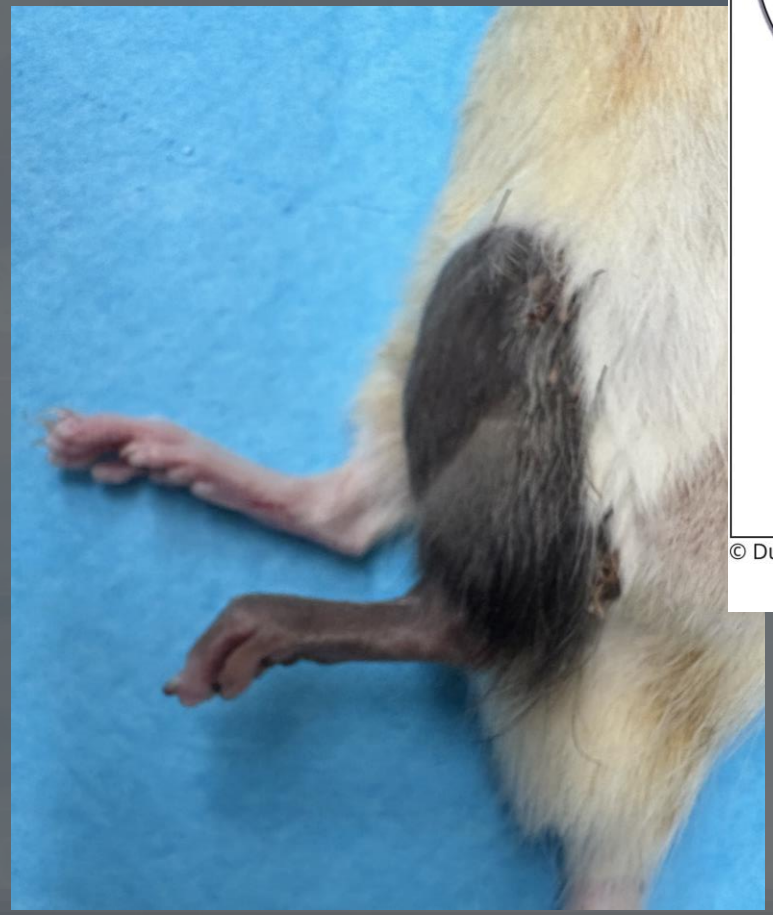
Hypothesis

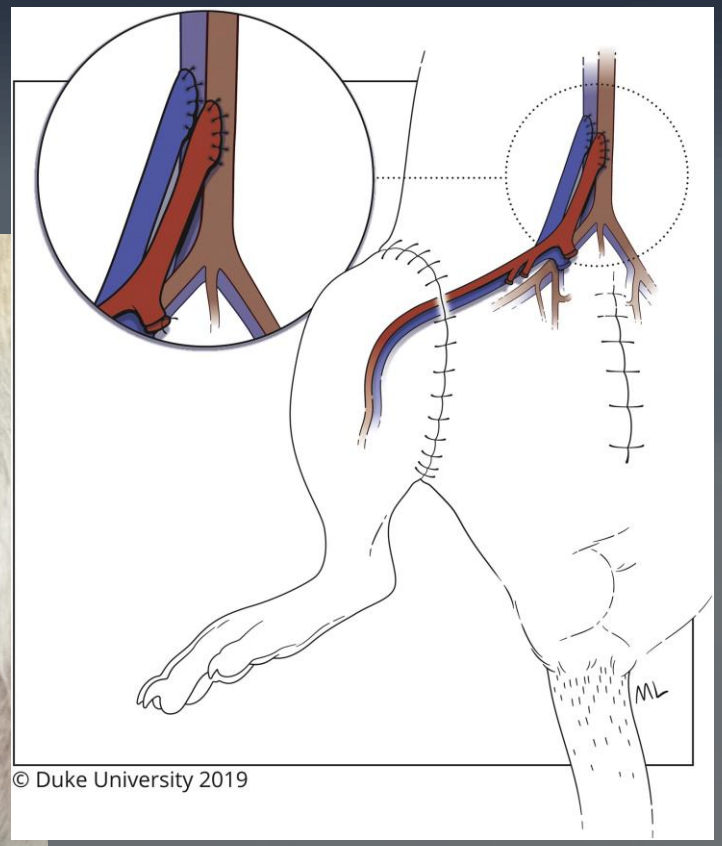
- Local calcitriol injection prolongs VCA graft survival by expanding regulatory T cells in peripheral blood and local lymphoid tissue, while minimizing systemic toxicity.

Mario G. Solari, MD

Methods

- MHC-mismatched BN → Lewis orthotopic hind limb transplant model,
- Standard tacrolimus (0.5 mg/kg) achieves indefinite graft survival (>140 days).
- Dose reduction to 0.1 mg/kg results in rejection at a mean of 43 days: a window where immunologic adjuncts can be tested.

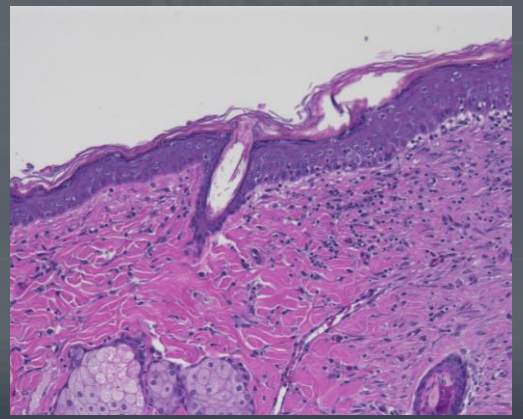




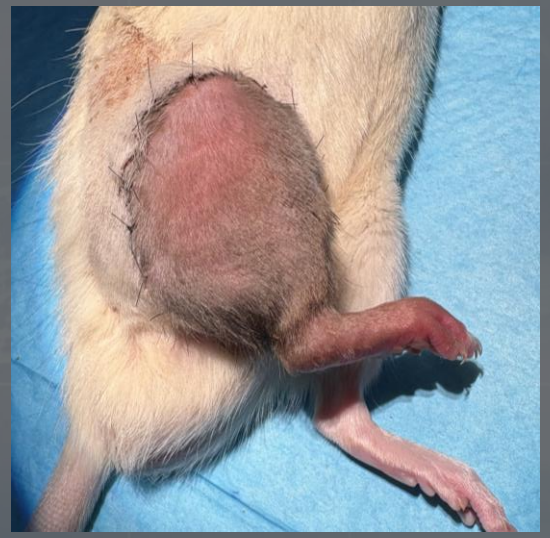
Vernon R, et al. J Surg Res. 2020;250:119–124.

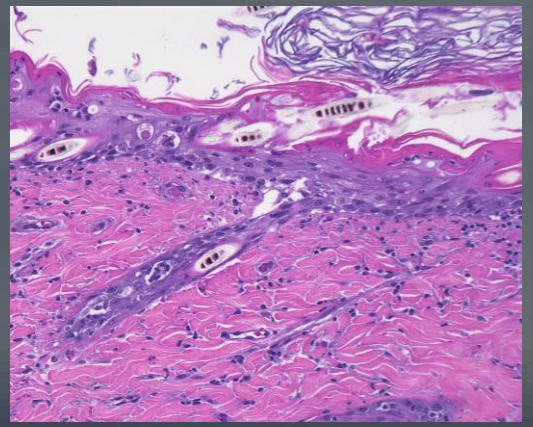
Acute Rejection in hind limb transplant model

Healthy

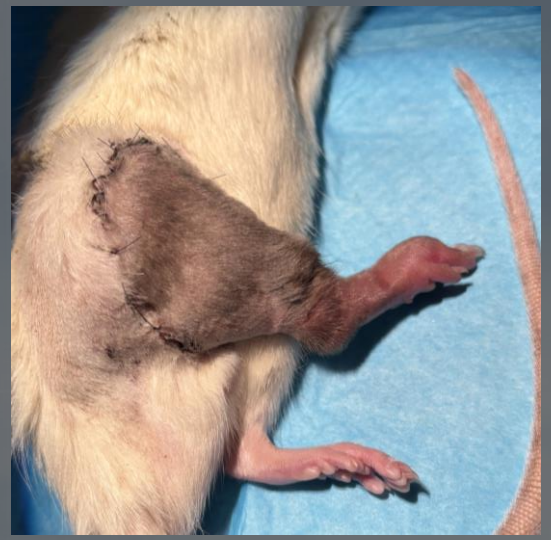


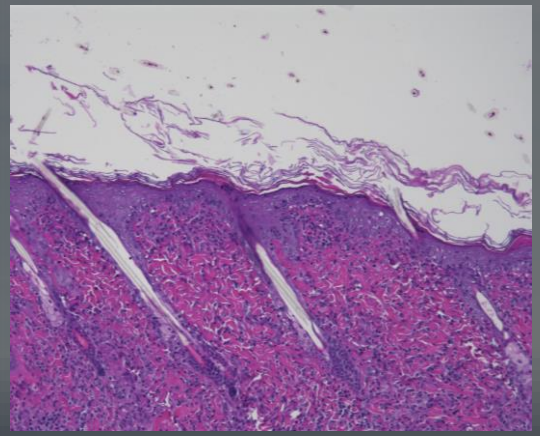
Grade 1 rejection



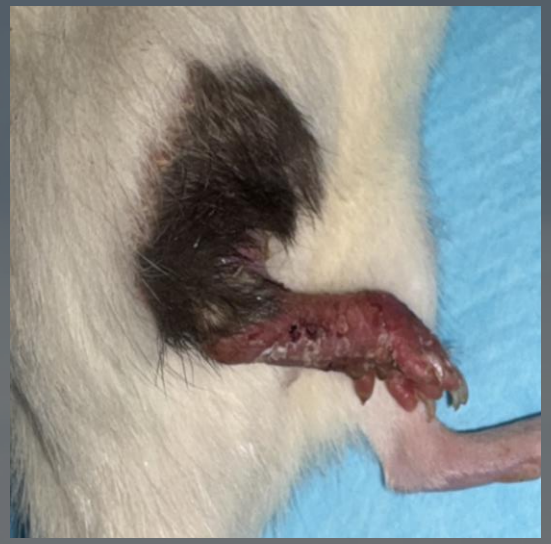


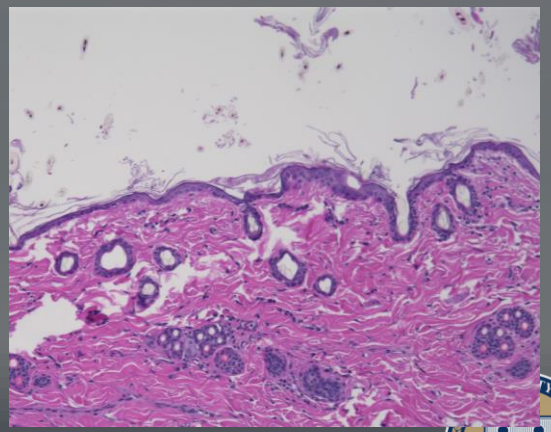
Grade 2 rejection





Grade 3 rejection





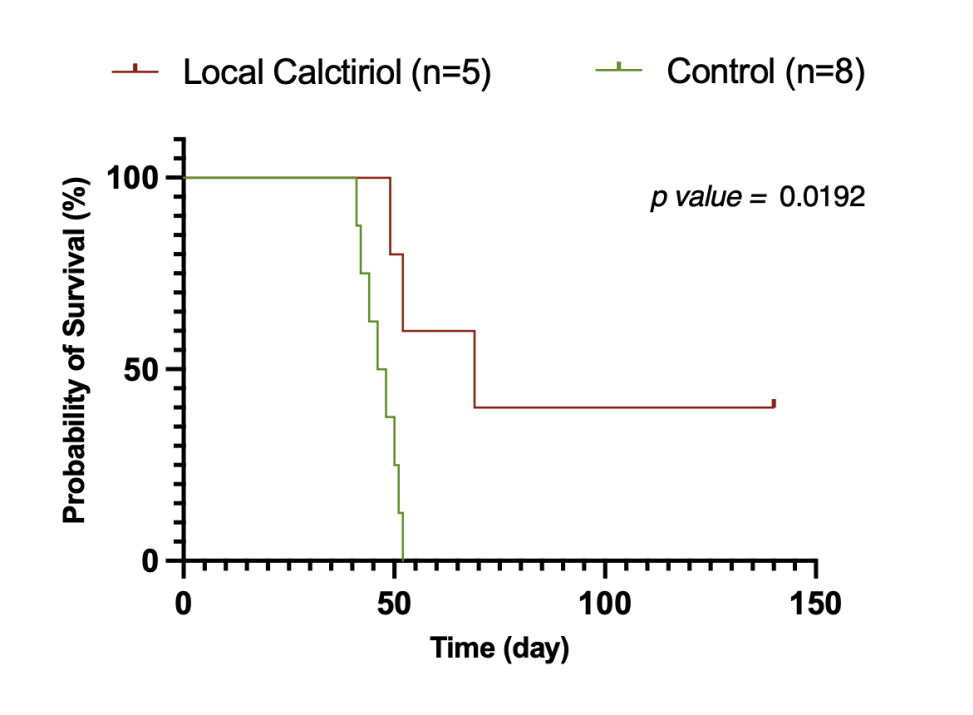
Study Groups

Following orthotopic hind limb transplantation, all recipients received high-dose tacrolimus (0.5 mg/kg/day) for 14 days to ensure initial engraftment, after which animals were randomized into three experimental groups:

Group	Intervention
Control (n=8)	*Low-dose tacrolimus (FK506 0.1 mg/kg/day)*
Local calcitriol (n=5)	*Low dose daily tacrolimus + local calcitriol (2 ug/kg) three times a week*
Positive control (n=5)	*High-dose tacrolimus (0.5 mg/kg) daily; indefinite survival benchmark (>140 days)*

Local calcitriol significantly prolonged VCA allograft survival compared with controls

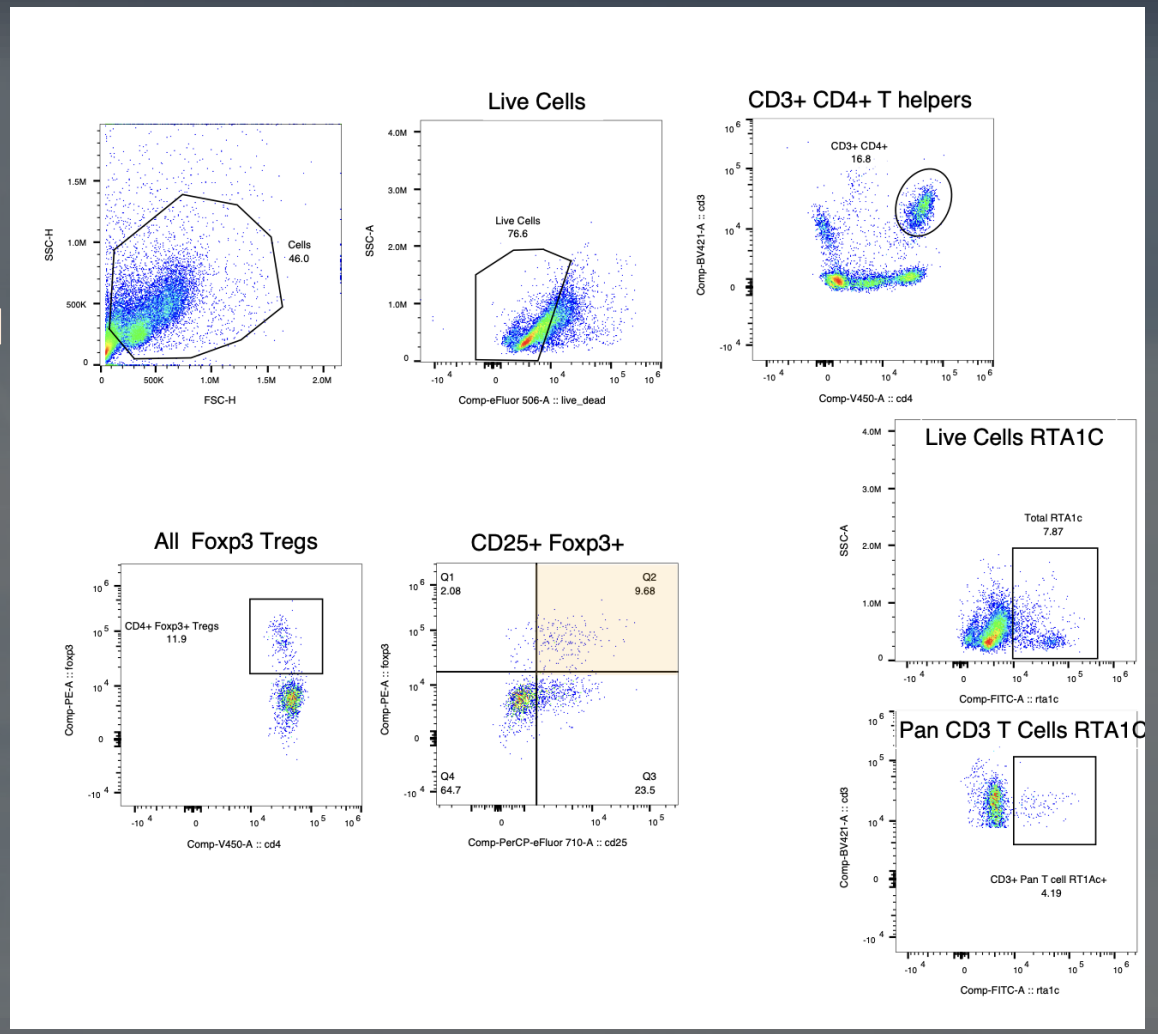
Mean survival was ***56.7 days*** (n = 5; range 49–69) in the calcitriol group vs ***46.3 days*** (n = 8; range 41–52) in controls (p = 0.015), with two calcitriol-treated animals remaining rejection-free at the 140-day endpoint.



Flow Cytometry

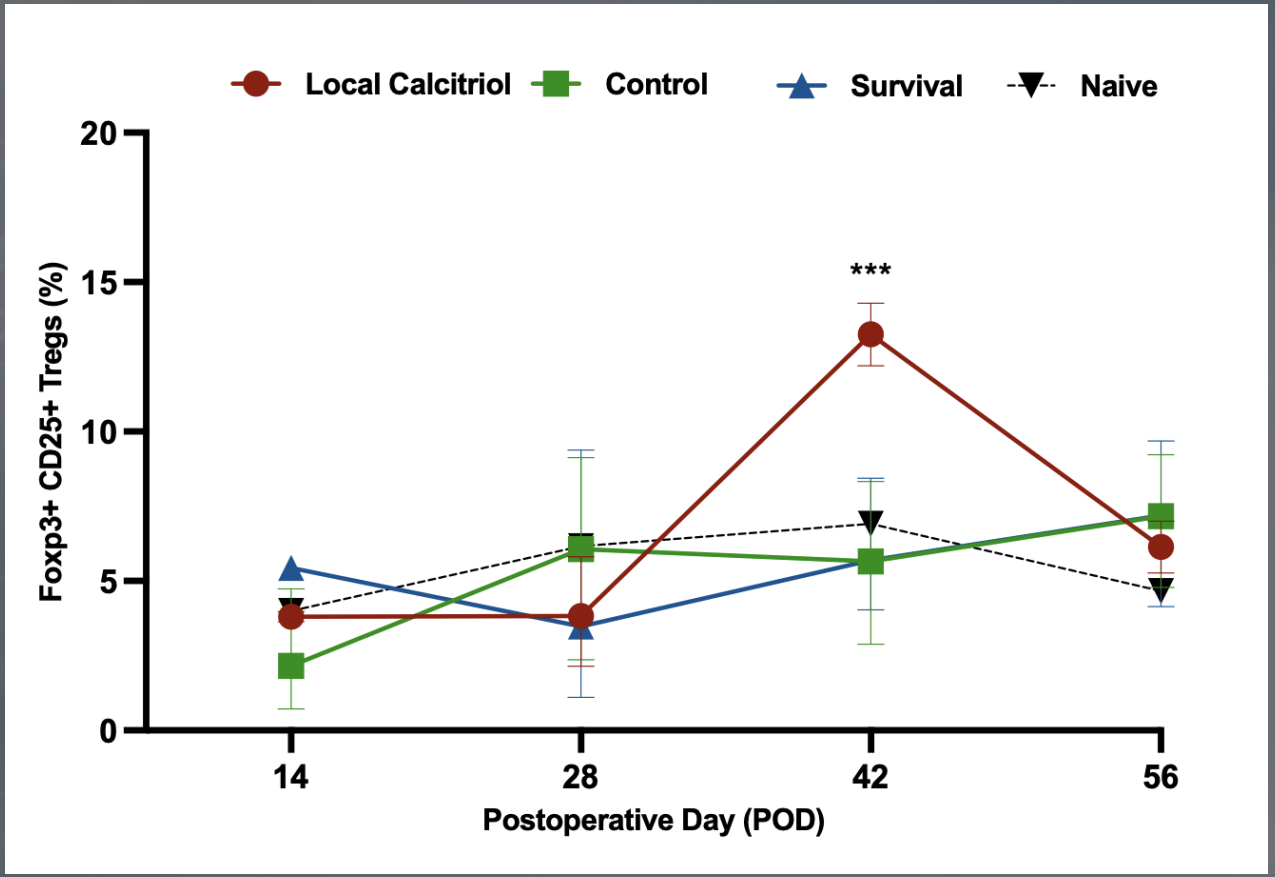
- Peripheral blood was collected biweekly.
- Immune cells were isolated using a standardized protocol and stained with extracellular and intracellular markers to quantify *Treg* populations and *chimerism* levels.

- (+ viability dye, + Fc blocker)
    - CD3
    - CD4
    - CD25
    - CD125
    - RTA1c
    - Foxp3



Treg Results in Blood

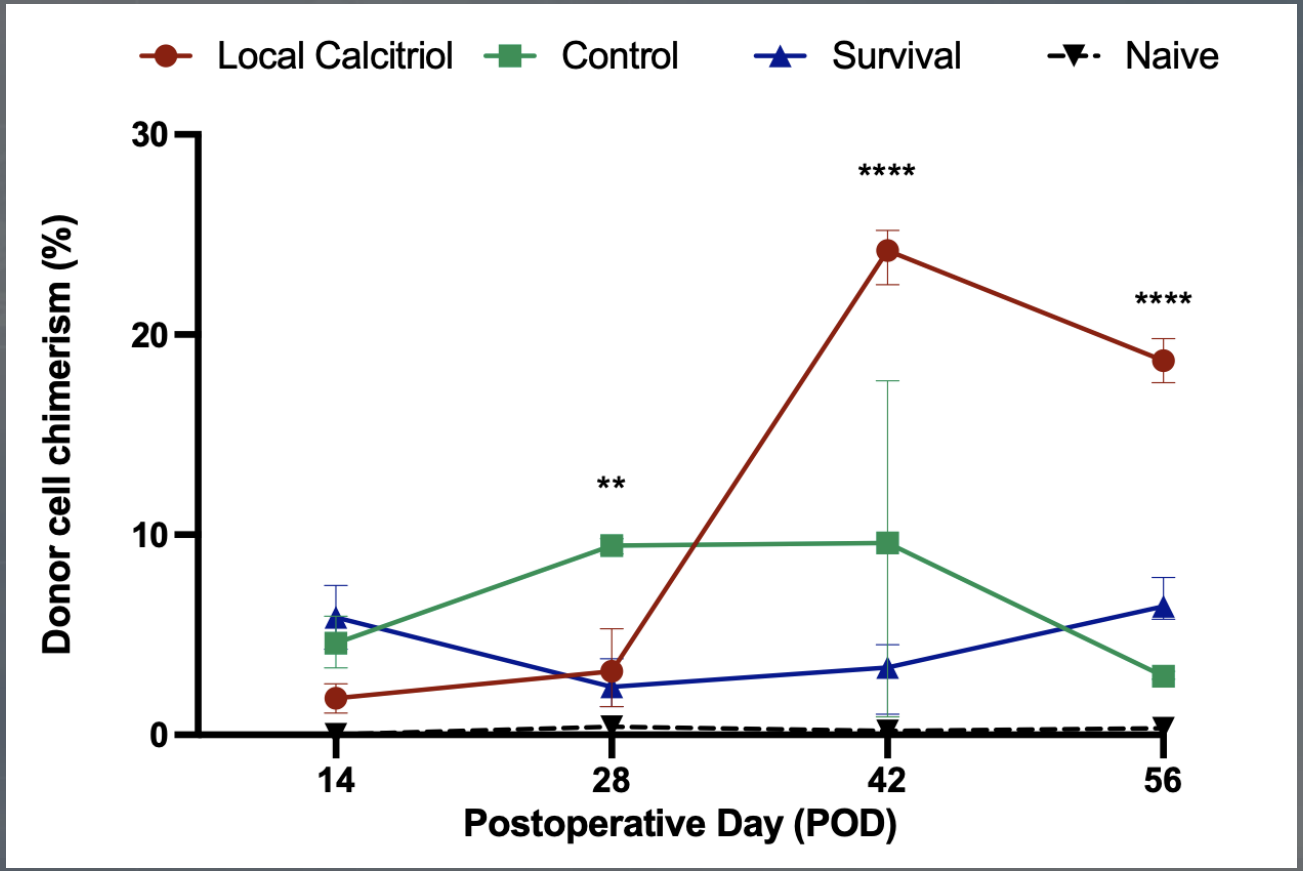
Local Calcitriol significantly increased FOXP3+ regulatory T-cell frequencies in the blood on POD 42.



Frequency of Tregs reported as (%) = (FOXP3$^+$ CD25$^+$ CD4$^+$ CD3$^+$ cells ÷ CD4$^+$ CD3$^+$ cells) × 100
Two-way ANOVA with Tukey's multiple comparisons test was used.
Non-rejected rats are excluded.
Error bars=SD

Chimerism Results in Blood

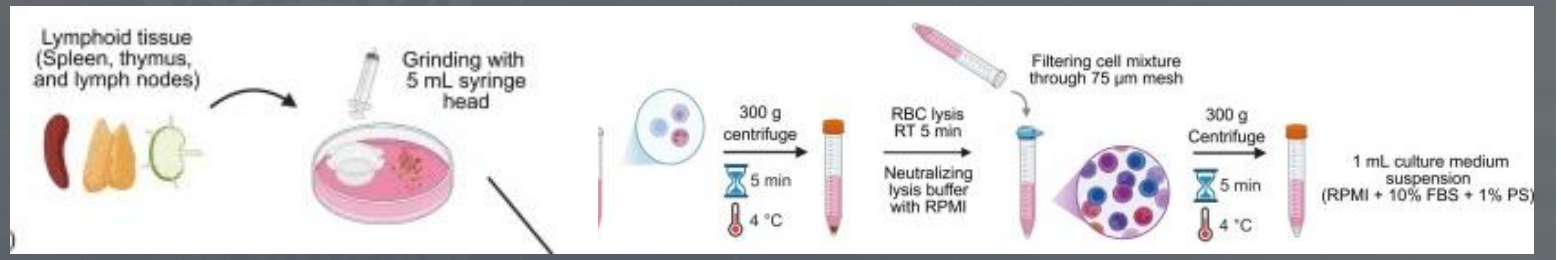
Local Calcitriol significantly increased donor cell chimerism (RTA1c) frequencies in the blood on POD 42 and POD 56 compared to low-dose tacrolimus only ($p<0.002$).



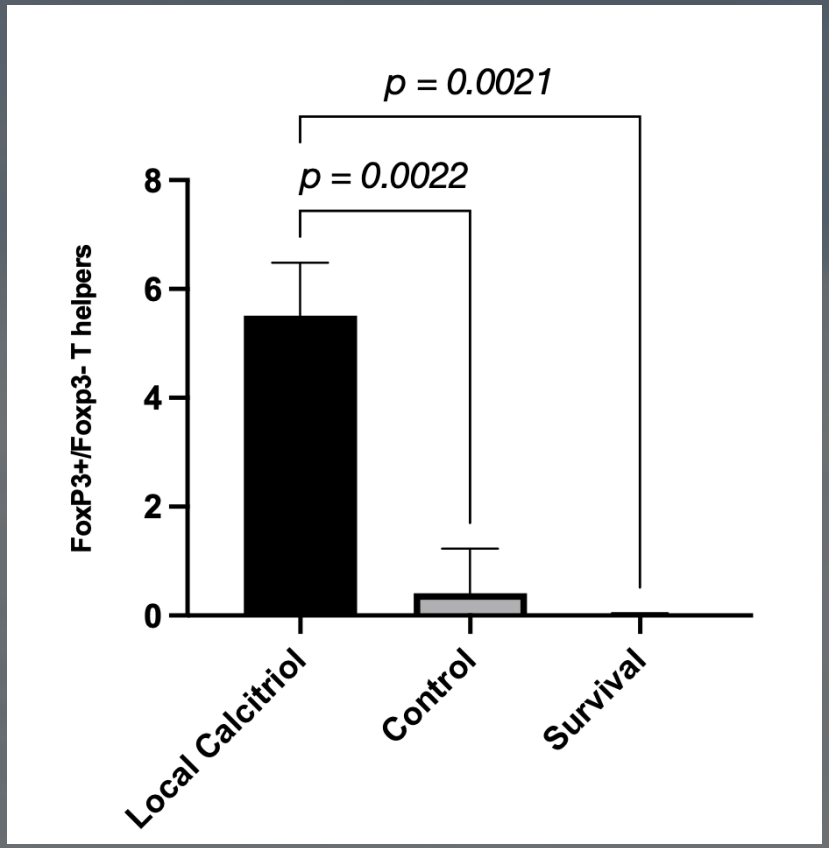
Donor chimerism (%) = (RTA1c$^+$ cells among live cells) × 100
Two-way ANOVA with Tukey's multiple comparisons test used.
Non-rejected rats are excluded.
Error bars=range

Treg Levels in Graft Lymph Node

Spleen and donor inguinal/popliteal lymph nodes were harvested for immune cell isolation.
Treg markers in donor popliteal nodes were significantly higher with local calcitriol than with low-dose tacrolimus alone or survival controls (both p<0.002).



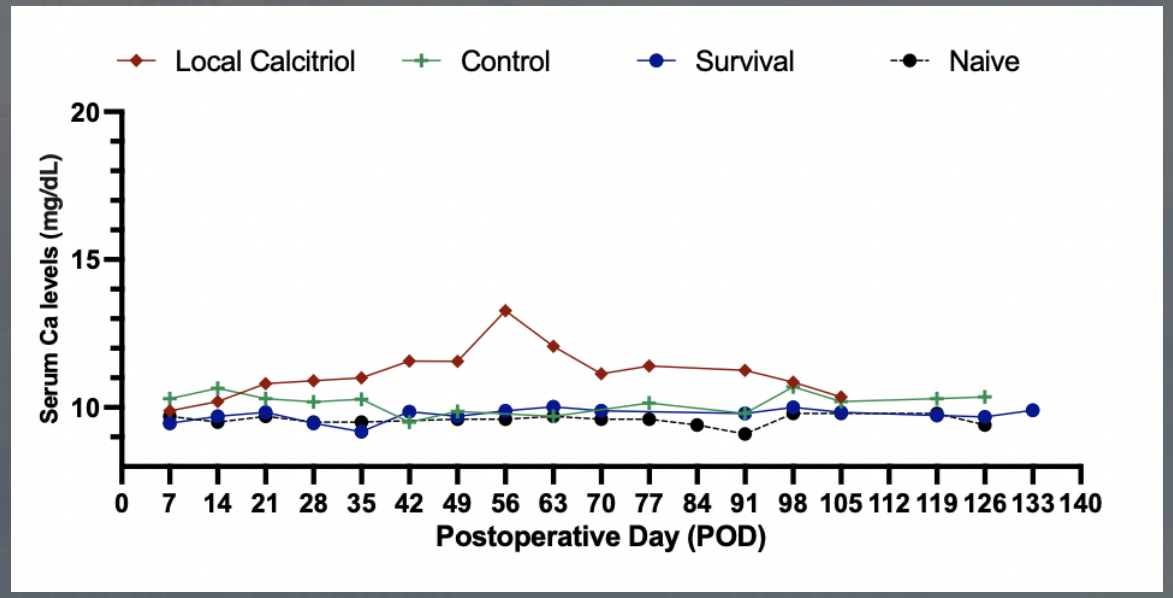
Park, J. S., & Lee, Y. *Molecules and cells*, 2025



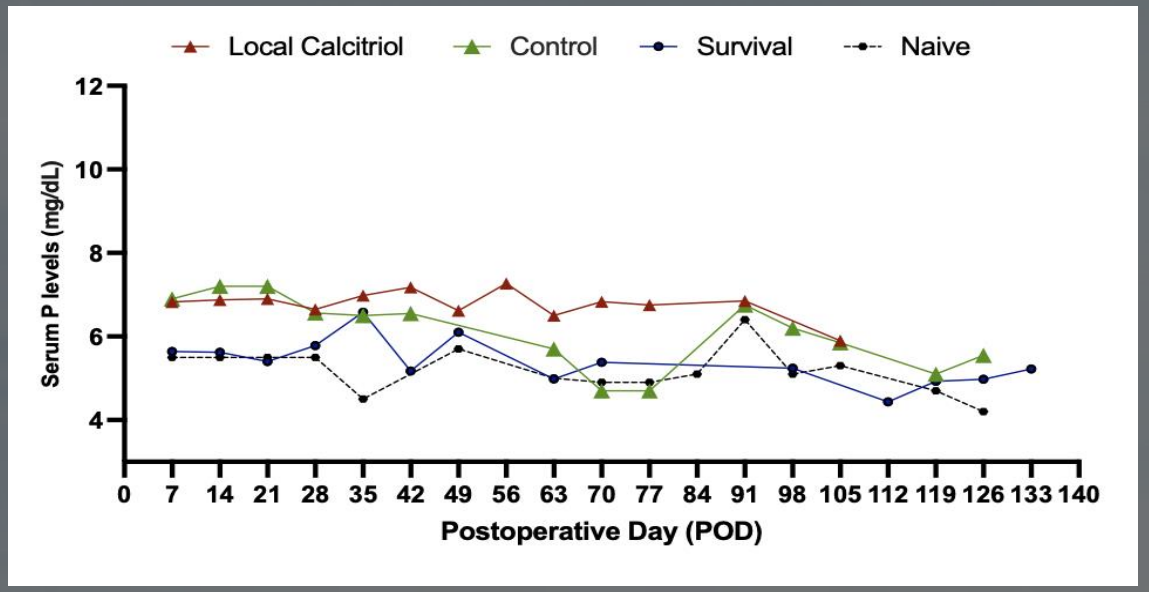
n = 8 (Control), 3 (Local calcitriol), 5 (Survival)
Two-way ANOVA with Tukey's multiple comparisons test used.
Rats who have accepted the graft are excluded.

Safety

The local calcitriol group showed a transient Ca peak of ~14 mg/dL at POD 56 — approaching but not sustaining hypercalcemia before returning to baseline. Phosphorus was mildly but acceptably elevated throughout.



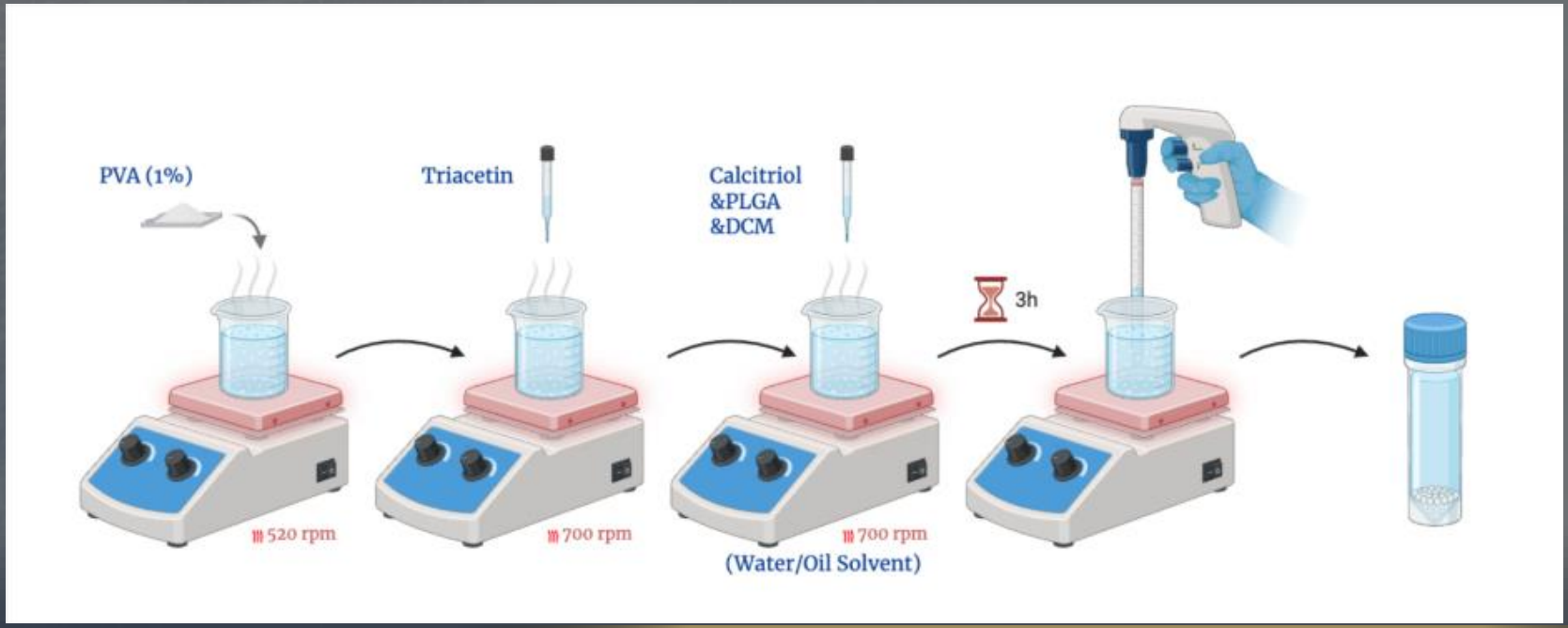
Ca levels



P levels

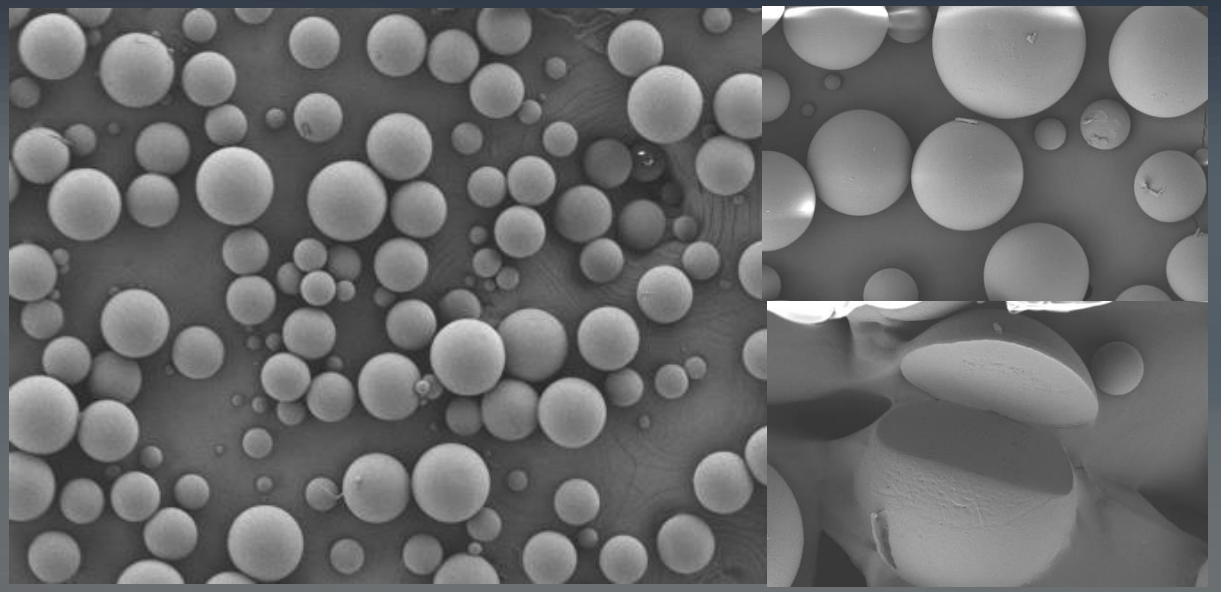
Novel calcitriol microspheres

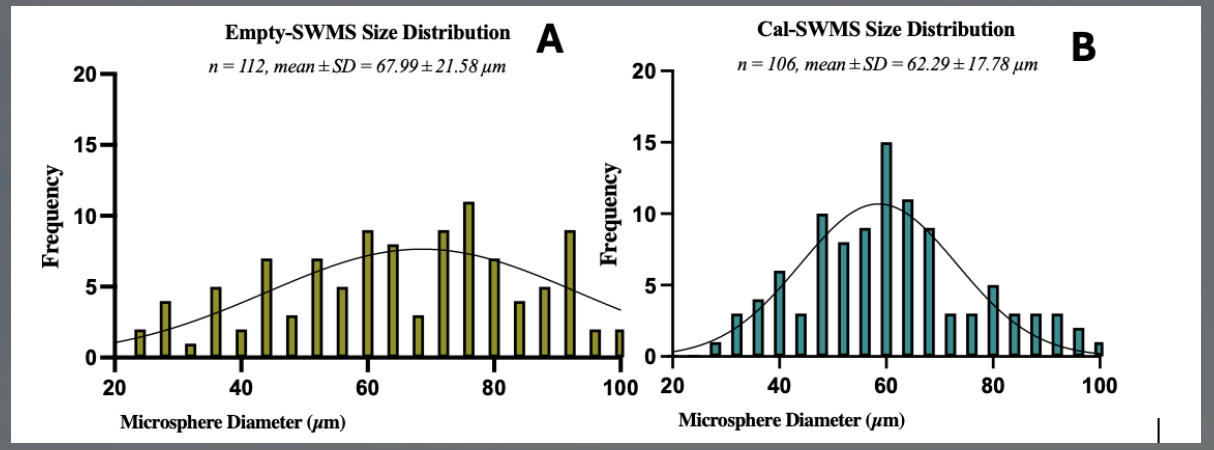
- To develop a locally deliverable, sustained-release calcitriol microsphere (MS) formulation that enables controlled, site-specific immunomodulation while minimizing systemic exposure.
- Calcitriol-loaded microspheres were fabricated using an **oil-in-water** emulsion solvent evaporation technique:



Calcitriol Microspheres

- SEM confirmed smooth, spherical microsphere morphology.
- Particle diameter analysis revealed a consistent size distribution with mean diameter of 62 µm.

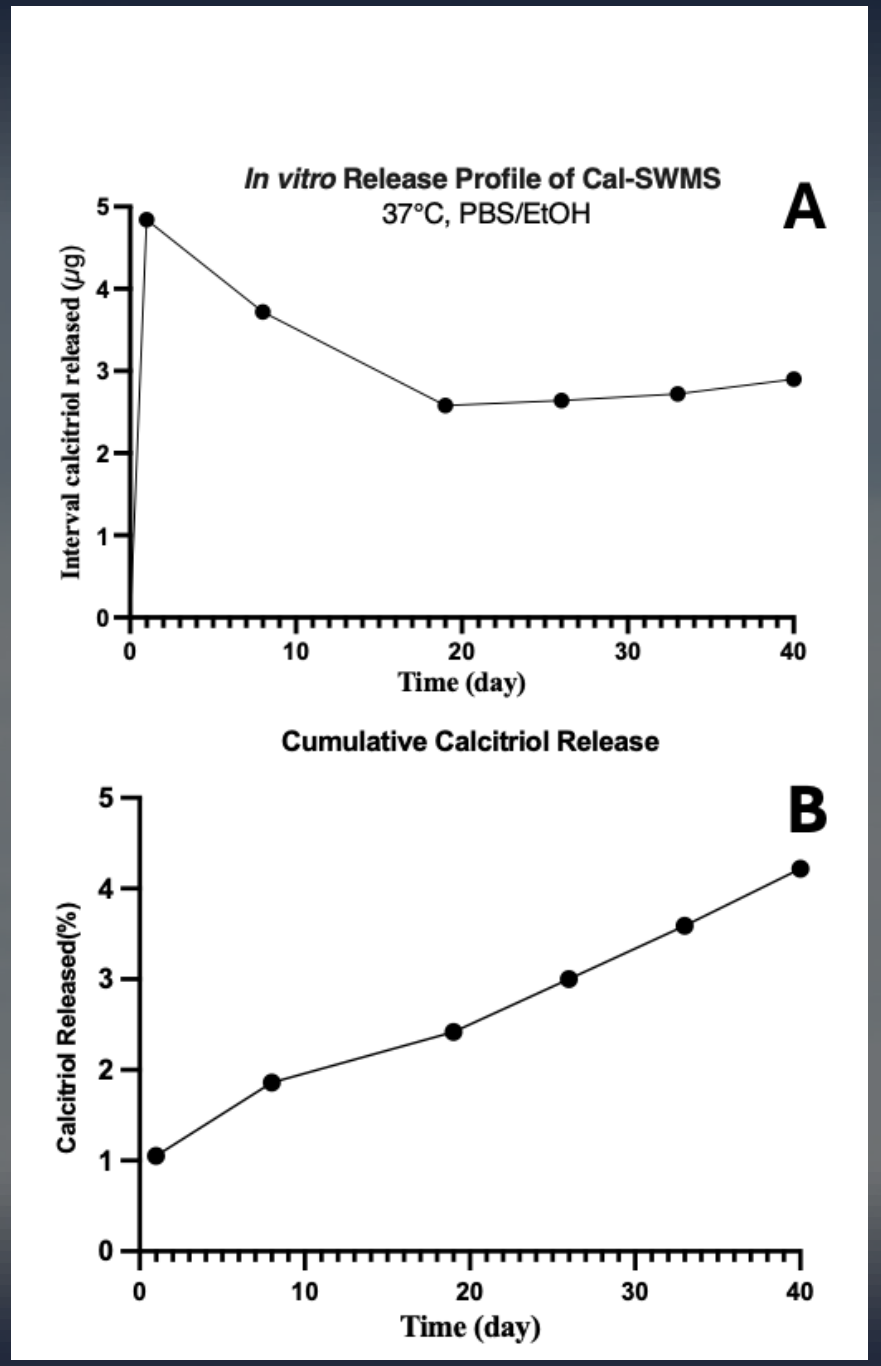




Controllable release of calcitriol

- An initial burst release of ~4.9 µg was observed at day 1, followed by a **sustained release of ~2.6–3.0 µg** per interval through day 40.

- Cumulative in vitro release reached ~4.2% by day 40, confirming a slow, sustained-release kinetic profile, (potentially) suitable for long-term local immunomodulation at the graft site.



Predicting Acute Rejection in VCA

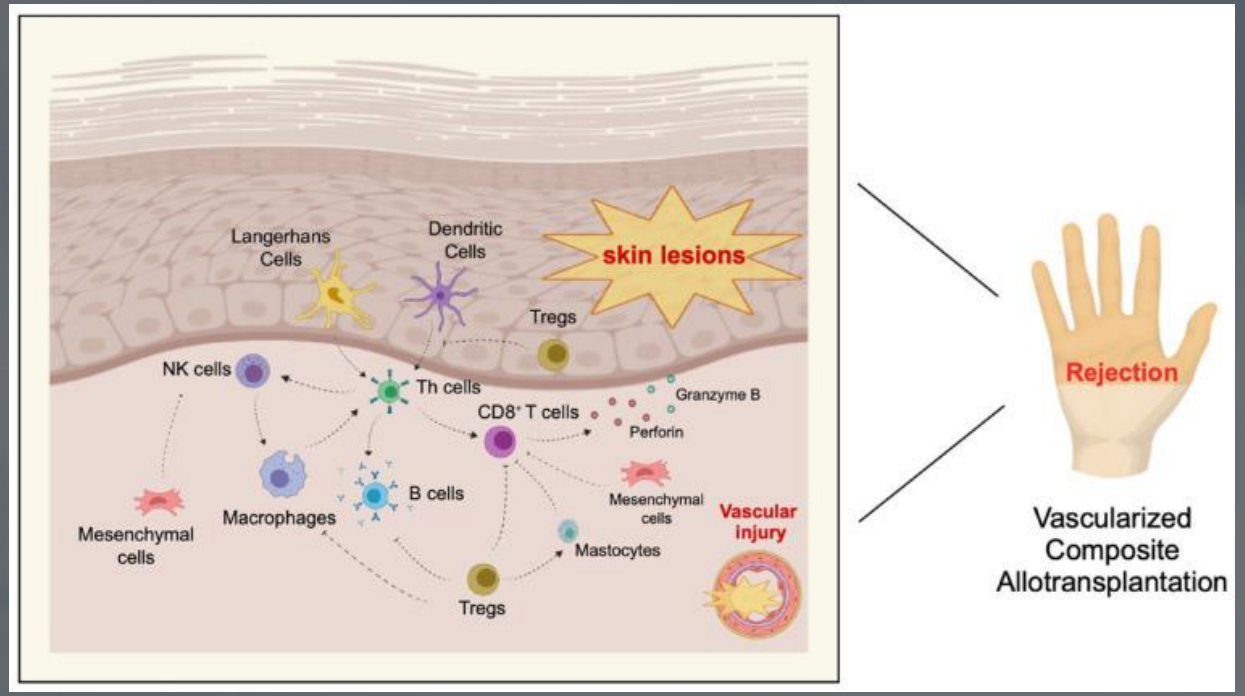
Punch biopsy with Banff grading remains the only diagnostic tool: invasive, reactive, and always one step behind.

Irreversible pathological changes occur prior to the onset of dermal findings; clinical intervention is frequently delayed

Predicting rejection before it happens is the most critical unmet need in VCA.

Background

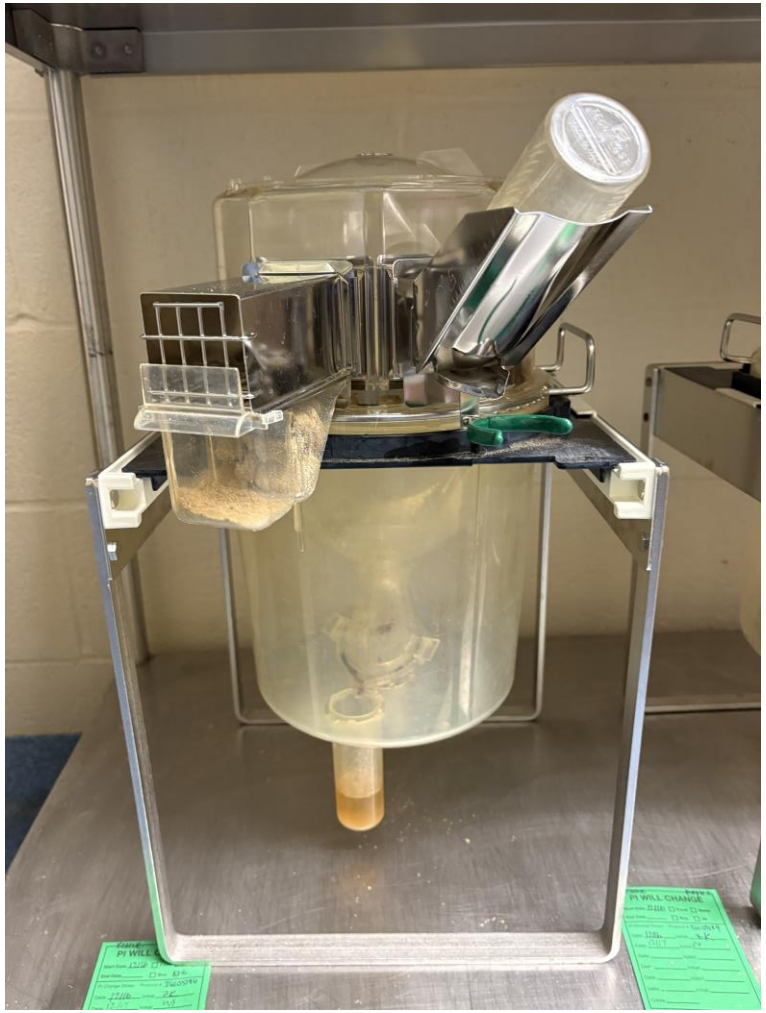
- There are many biomarkers (blood, urine) that has been researched on solid organ transplant rejection.
- VCA is fundamentally different: composite tissue immunogenicity, skin-dominant rejection
- Biomarkers validated in solid organ transplantation cannot be directly applied to VCA



Ren et al., Journal of Tissue Engineering, 2024

Methods

- BN → Lewis hind limb transplant model used as the VCA platform
- Recipients housed in metabolic cages for 24-hour urine collection at defined postoperative timepoints
- Samples cryopreserved and biobanked for future *non-targeted Mass Spectrometry proteomics analysis:* enabling unbiased discovery of rejection-associated urinary biomarkers



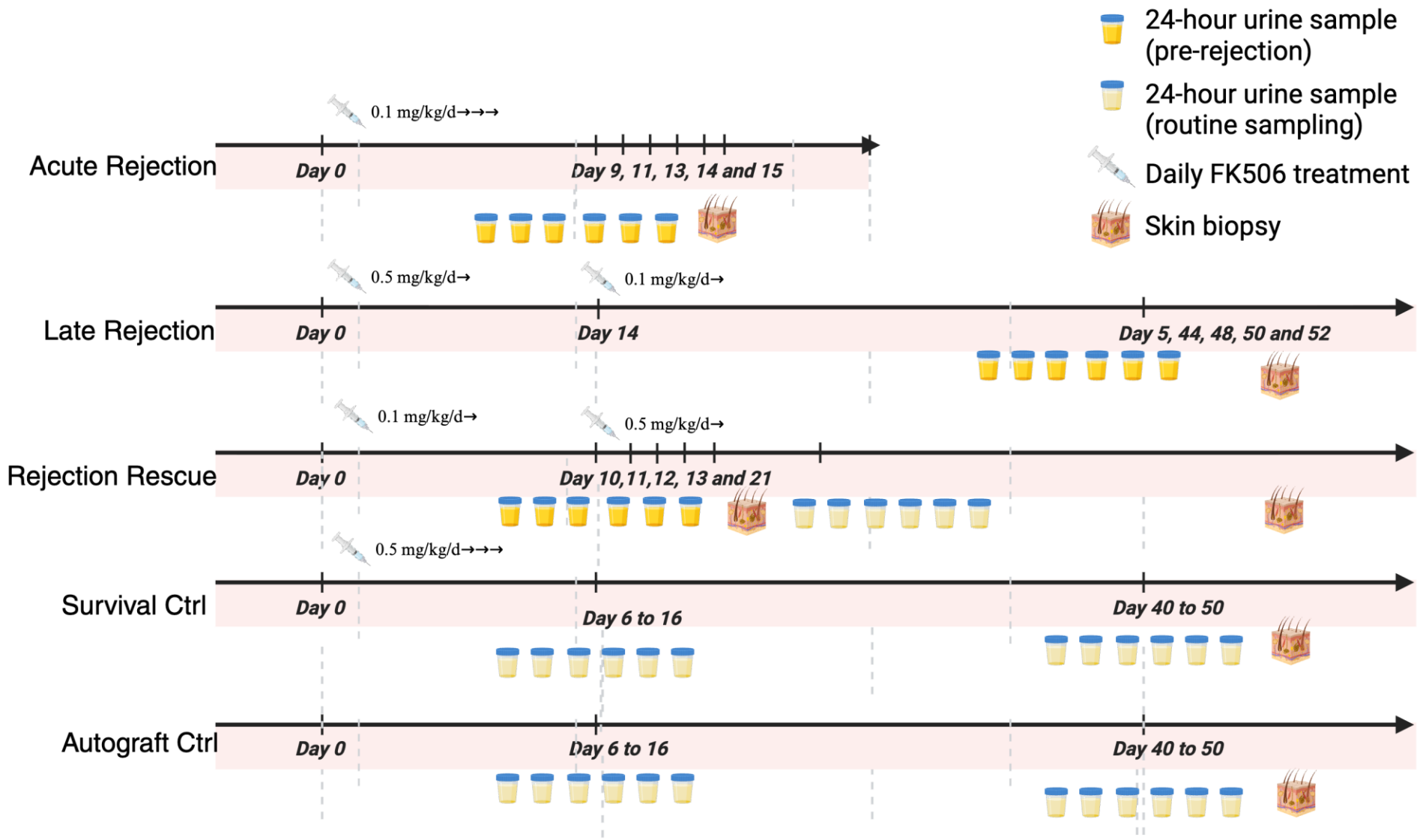
Study Groups

Five groups with distinct survival trajectories established to capture the full spectrum of rejection patterns:

Group	Intervention	Graft survival (POD)
Acute Rejection	0.1 mg/kg tacrolimus daily	13
Late Rejection	0.5 mg/kg for 14 days, followed by 0.1 mg/kg tacrolimus daily	43
Autograft ctrl	-	140
Survival ctrl	0.5 mg/kg tacrolimus daily	140
Rejection rescue	0.1 mg/kg tacrolimus until grade 1 rejection, followed by 0.5 mg/kg daily	13-50*

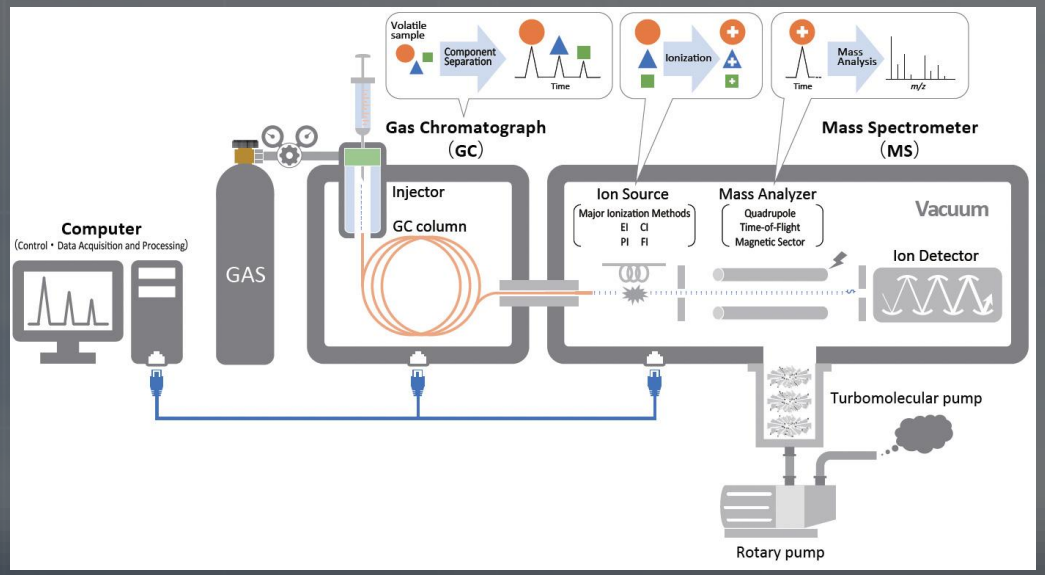
*Rejection onset on low-dose tacrolimus, subsequently halted by dose escalation

Experimental Design and Sampling Timeline in VCA Model



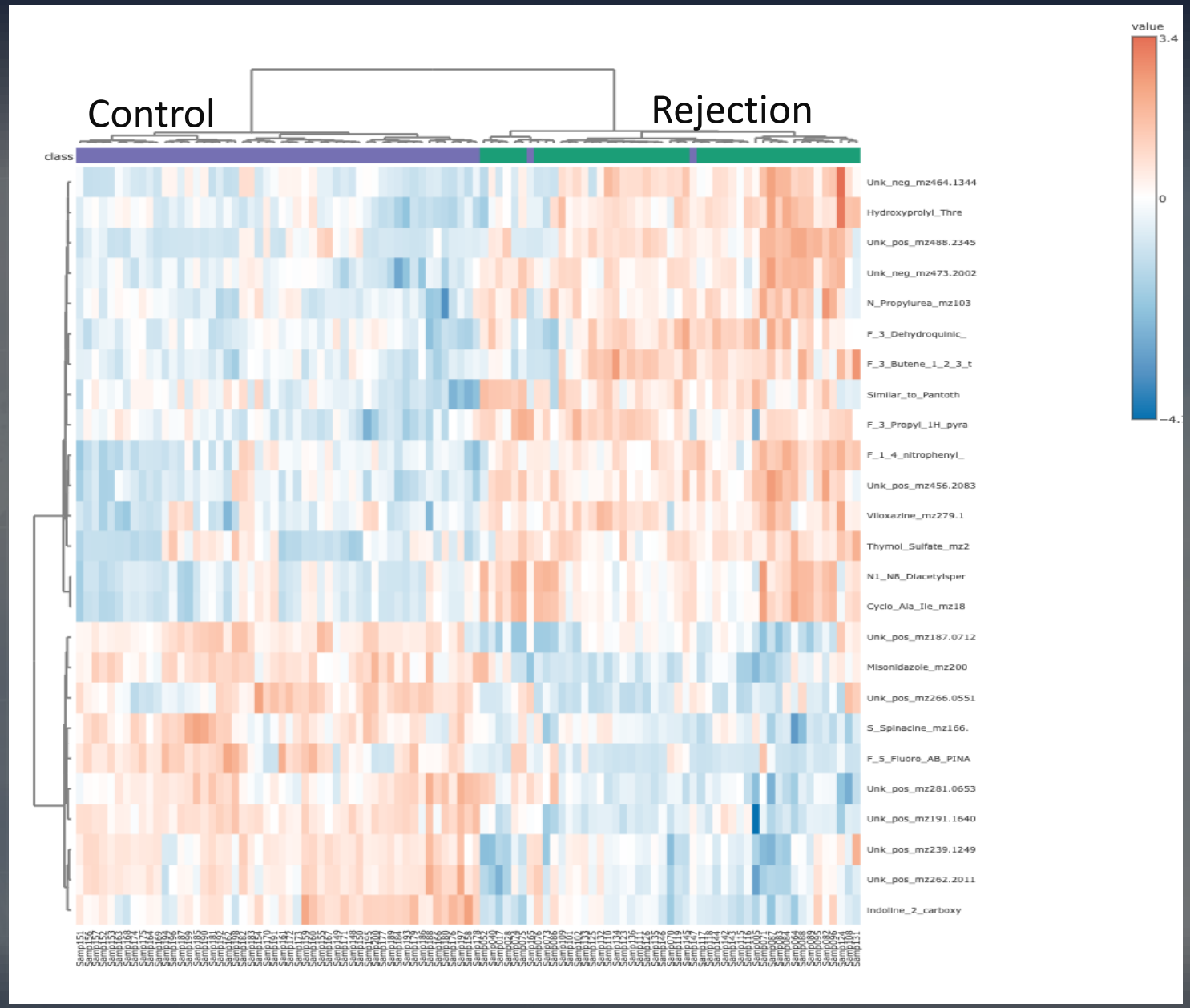
Samples

- **<u>1,500+ urine samples</u> collected to establish biobank**
- Of which 200 critical samples spanning key rejection timepoints were transferred to the Mass Spectrometry Core for non-targeted proteomic analysis.



Raw Data:

- Started with 5,889 detected metabolites across 200 urine samples plus 14 pooled QC (quality control) samples run alongside them which are pooled mixtures of the actual study samples, injected repeatedly throughout the run.
- Compound Discoverer software detected and quantified features: MS core did this part

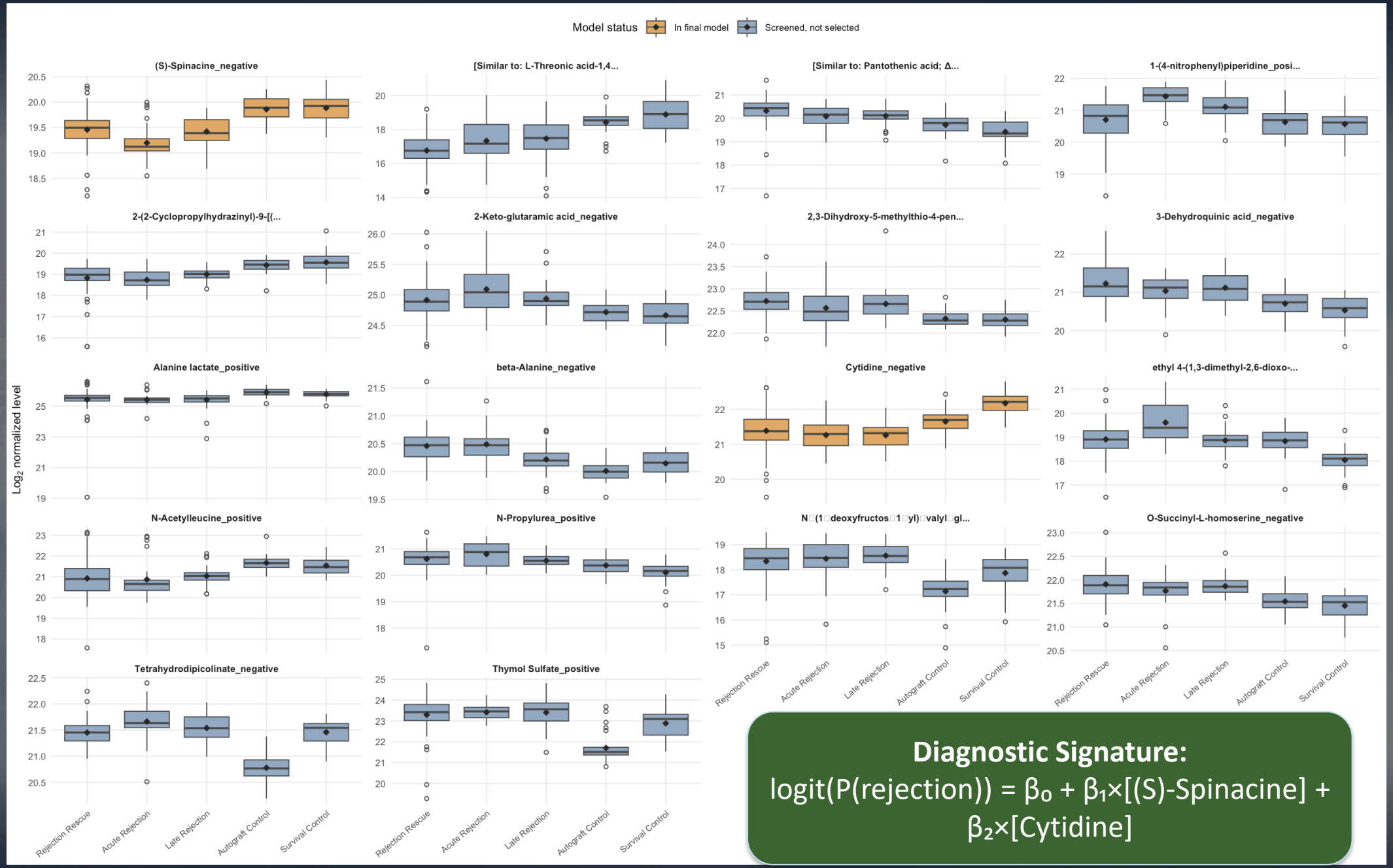

Control
Rejection
class
value
3.4
0
Unk_neg_mz464.1344
Hydroxyprolyl_Thre
Unk_pos_mz488.2345
Unk_neg_mz473.2002
N_Propylurea_mz103
F_3_Dehydroquinic_
F_3_Butene_1_2_3_t
Similar_to_Pantoth
F_3_Propyl_1H_pyra
F_1_4_nitrophenyl_
Unk_pos_mz456.2083
Viloxazine_mz279.1
Thymol_Sulfate_mz2
N1_N8_Diacetylsper
Cyclo_Ala_Ile_mz18
Unk_pos_mz187.0712
Misonidazole_mz200
Unk_pos_mz266.0551
S_Spinacine_mz166.
F_5_Fluoro_AB_PINA
Unk_pos_mz281.0653
Unk_pos_mz191.1640
Unk_pos_mz239.1249
Unk_pos_mz262.2011
Indoline_2_carboxy

Diagnostic Signature

Spinacine and cytidine performed better individually (higher AUC and log-likelihood), so those two ended up forming our final diagnostic signature instead.

$$\text{logit}(P(\text{rejection})) = \beta_0 + \beta_1 \times [\text{(S)-Spinacine}] + \beta_2 \times [\text{Cytidine}]$$

β_0 = the intercept
β_1 = the coefficient (weight) for Spinacine
β_2 = the coefficient (weight) for Cytidine



Diagnostic Signature:

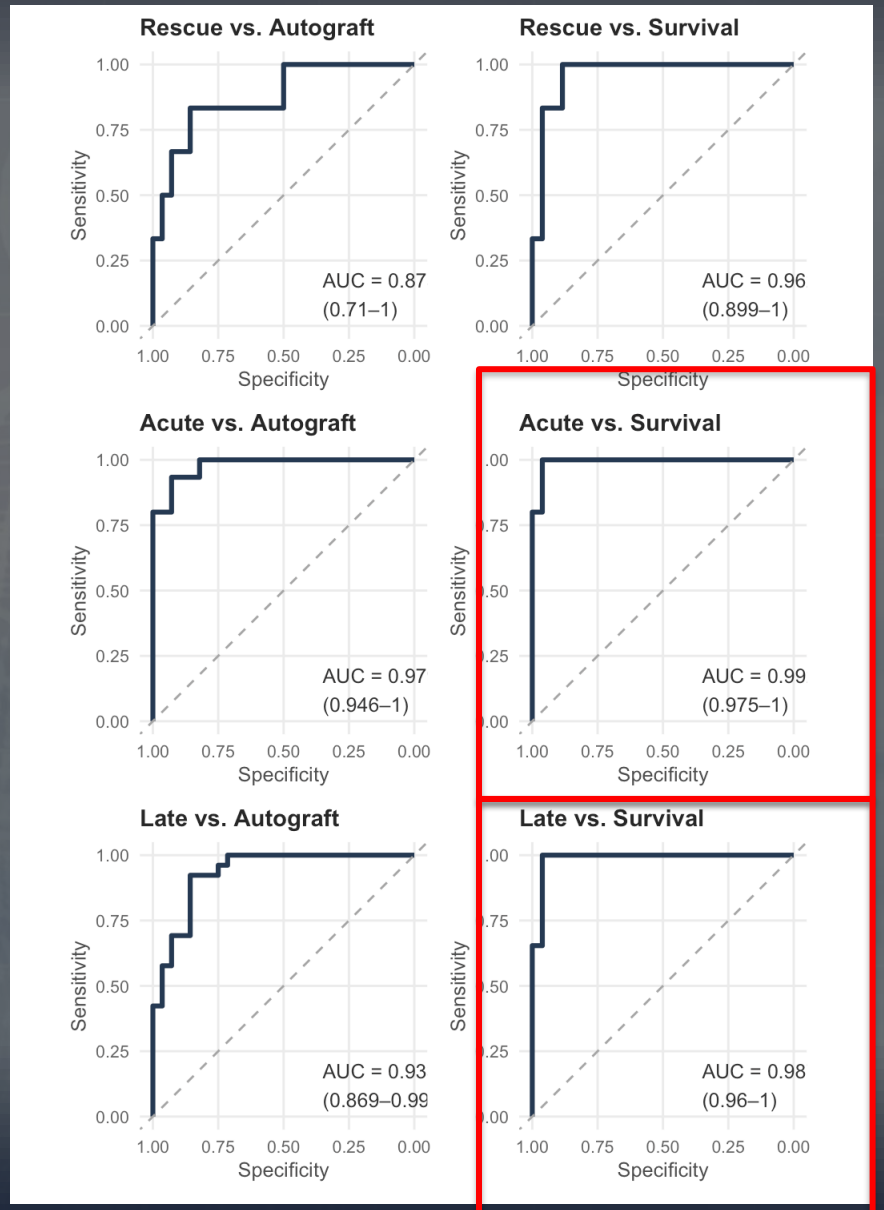
$$\text{logit}(P(\text{rejection})) = \beta_0 + \beta_1 \times [\text{(S)-Spinacine}] + \beta_2 \times [\text{Cytidine}]$$

Diagnostic & Predictive Power

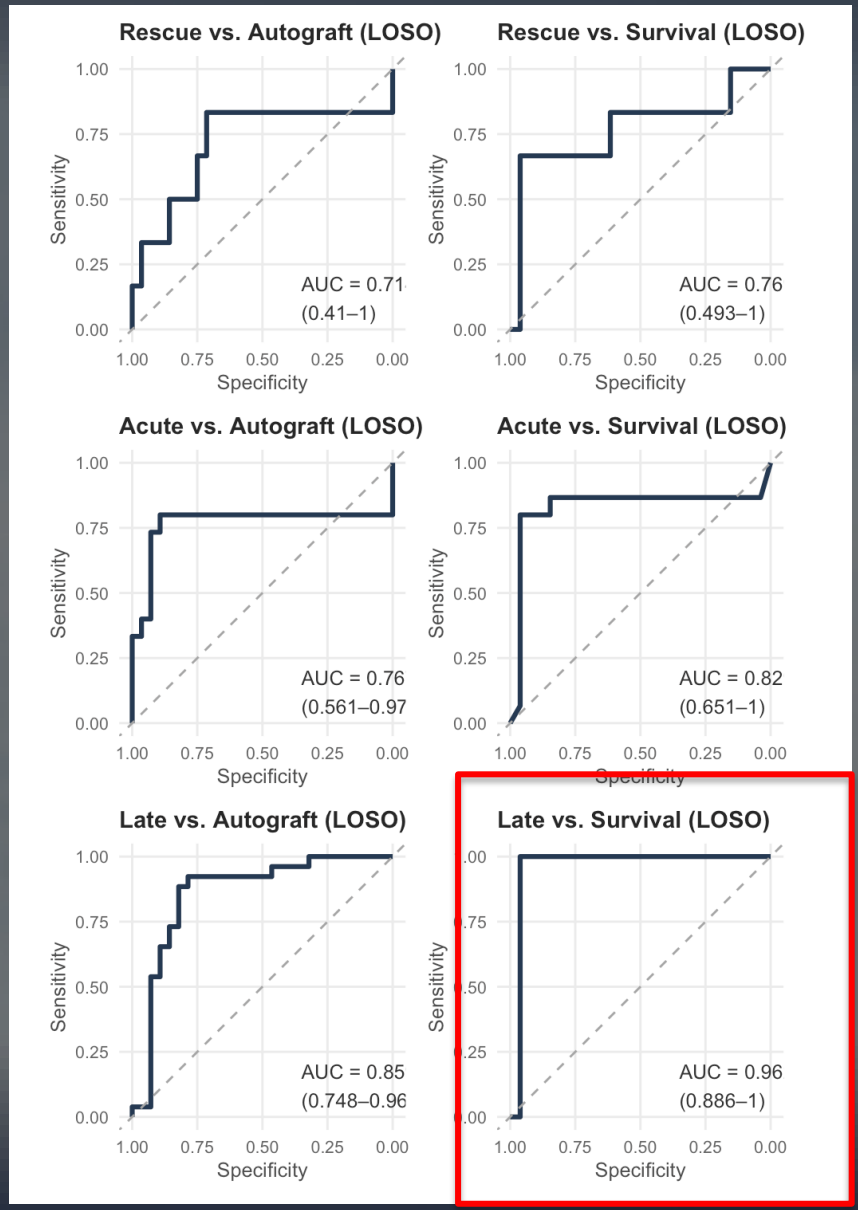
Case group: Active Rejection (Rejection Rescue + Acute + Late), grade 1-3 samples
Control group: Autograft + Survival Control combined

	AUC	*95% CI*	*Threshold*	*Sensitivity*	*Specificity*	*Youden's J*
Diagnostic	**0.962**	**0.930–0.993**	**0.297**	**0.936**	**0.889**	**0.825**
LOSO *cross-validated*	**0.933**	**0.883–0.983**	**0.395**	**0.894**	**0.889**	**0.783**
Predictive *(LOSO cross-validated)*	**0.921**	**0.866–0.977**	**0.266**	**0.892**	**0.870**	**0.762**

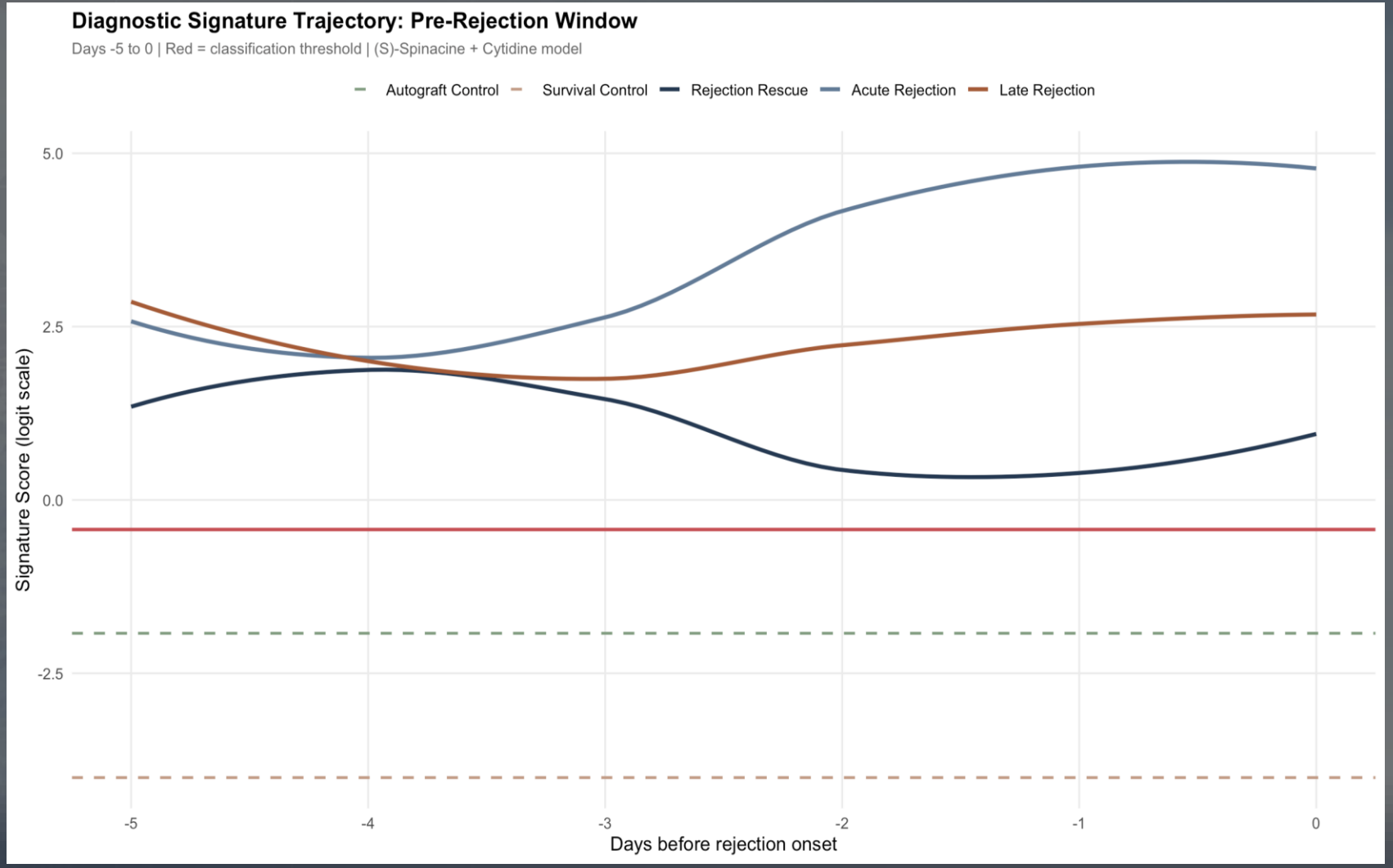
Diagnostic Power



Leave-One-Subject-Out



Predictive Analysis



Conclusion

Local calcitriol prolonged VCA graft survival, supporting a Treg-mediated tolerogenic mechanism as an adjunct to reduced-dose tacrolimus

Acute rejection in VCA can be detected non-invasively using a urine proteomics panel, five days before the first signs of dermal changes appear.

Local non-toxic drug delivery mechanisms should be further tested for their effectiveness in treating VCA immunosuppression.

Future Directions

In vivo pharmacokinetic characterization of Cal-SWMS to confirm local drug distribution and systemic spillover

Efficacy evaluation of Cal-SWMS in the BN → Lewis hind limb transplant model vs. empty microsphere and control groups to validate survival prolongation

Proof-of concept urine biomarker panel in VCA rejection to be tested on large animals and bigger cohorts

References

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Thank you

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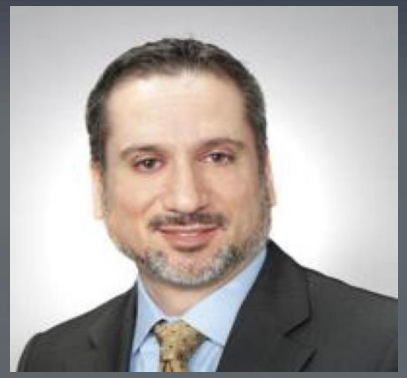

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