

# Gene Expression Patterns in Respiratory Hypersensitivity

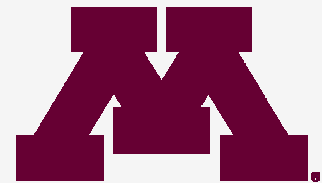
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**College of Veterinary Medicine**

**Department of Mathematics and Statistics**

**UNIVERSITY OF MINNESOTA**

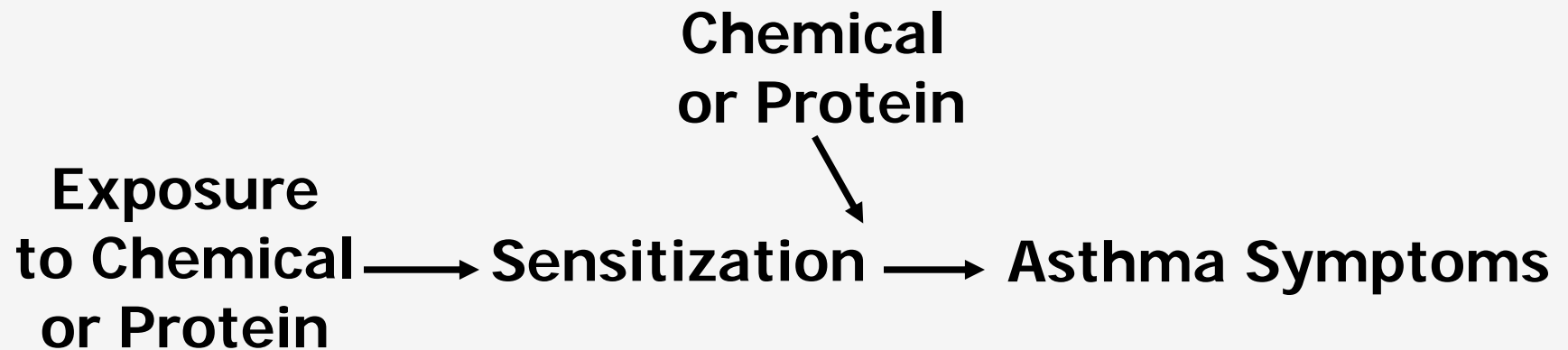


# Sensitization

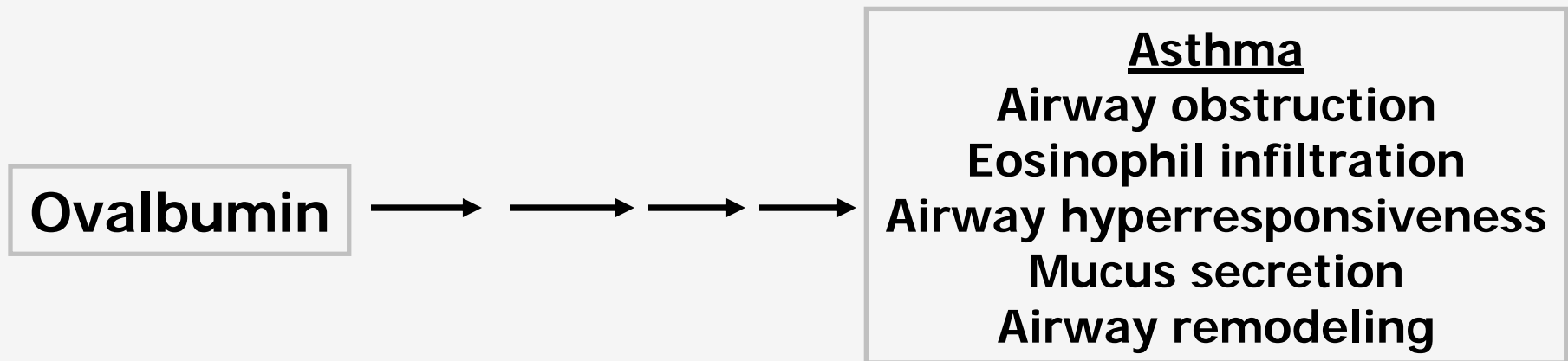
Induction Phase

# Challenge

Effector Phase



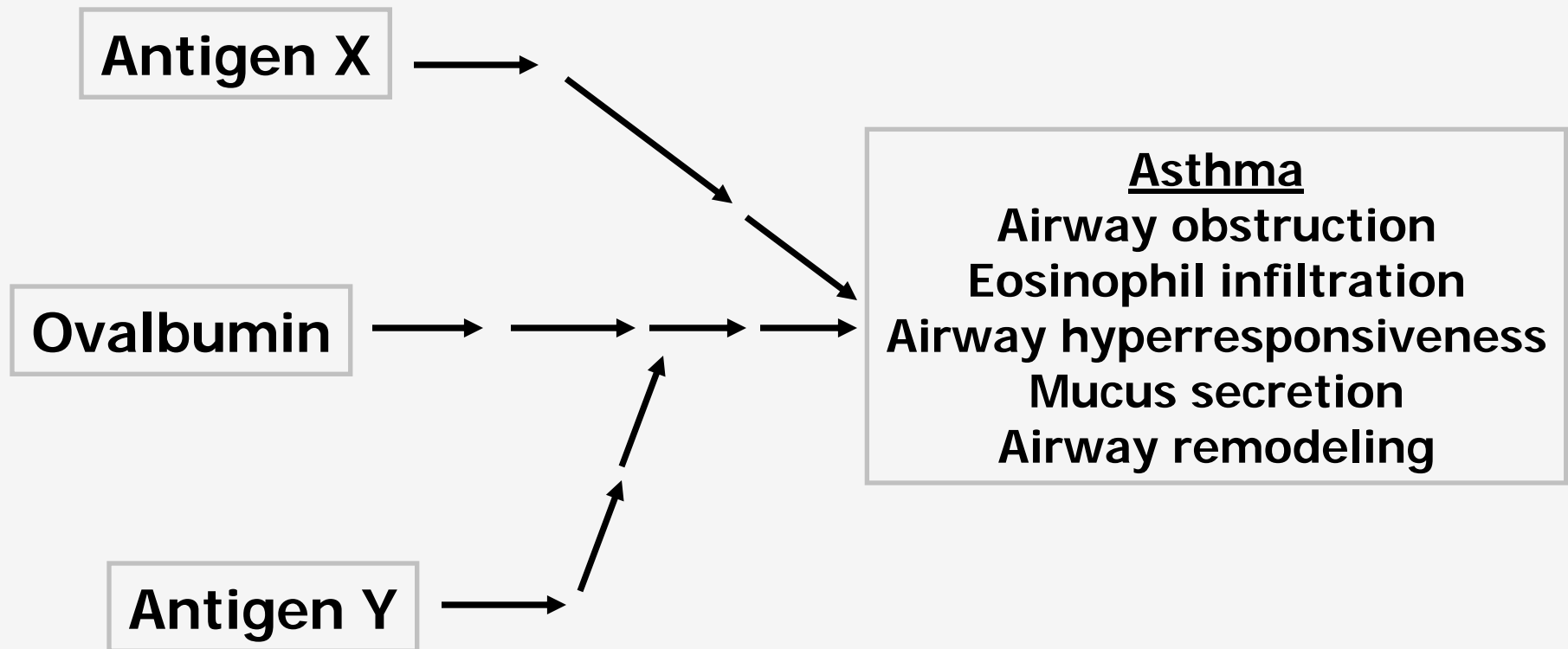
# Hypothetical Effector Pathways for Asthma



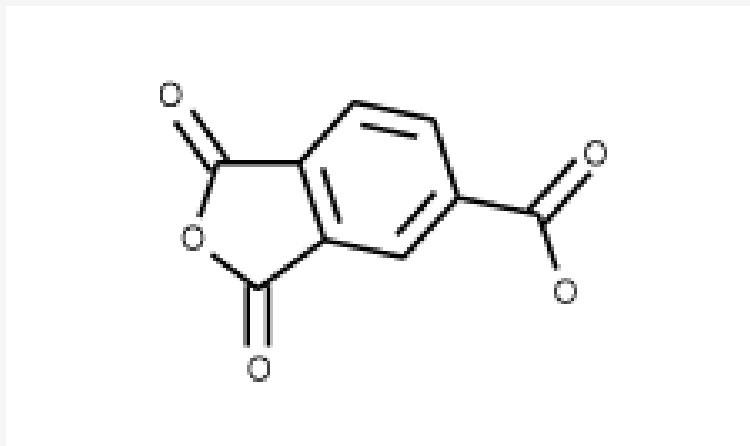
# Hypothesis

**Different antigens evoke unique effector mechanisms leading to the asthma phenotype**

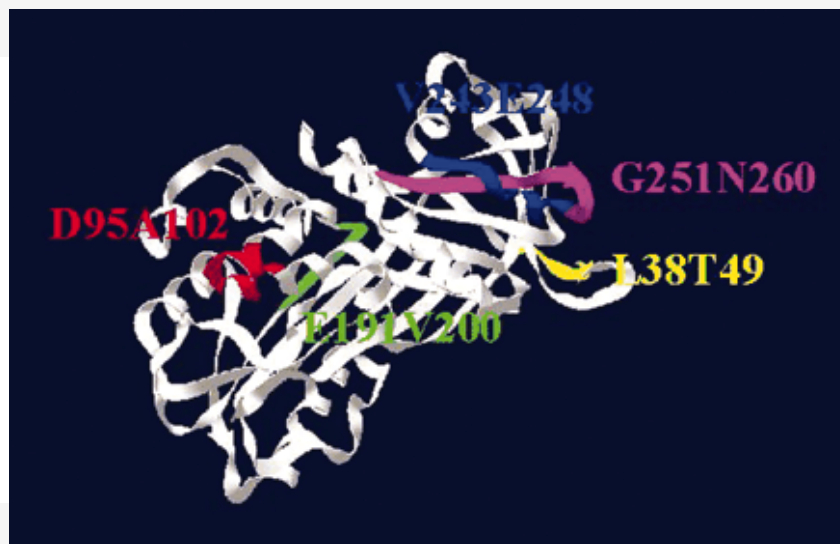
# Hypothetical Effector Pathways for Asthma



# Trimellitic Anhydride and Ovalbumin



**Trimellitic Anhydride**  
MW 192



Mine & Rupa (2003)

**Ovalbumin**  
MW ~45,000

# Why Ovalbumin and TMA?

- **Ovalbumin (OVA)**
  - Extensive information regarding effector mechanisms in murine asthma models using ovalbumin
  - Occupational allergen and reference protein allergen in immunology
- **Trimellitic anhydride (TMA)**
  - Known respiratory occupational allergen

# Specific Aim

**Identify differentially expressed transcripts in the lungs of mice sensitized and challenged with either ovalbumin (OVA) or trimellitic anhydride (TMA)**

**Unique patterns of gene expression with different allergens suggest unique effector mechanisms or reflect heterogeneity of asthma symptoms.**



# Experimental Design

- Genetically inbred mouse, BALB/c
- Sensitized and challenged by the same experimental protocol for both OVA and TMA
- Measurement of the Asthma phenotype
  - Eosinophil infiltration into the lung
- Affymetrix Microarrays of whole lung
  - MG\_U74Av2 array (>12,000 probe sets) or MG\_430 2.0 array (>45,000 probe sets)

# Why Affymetrix Arrays?

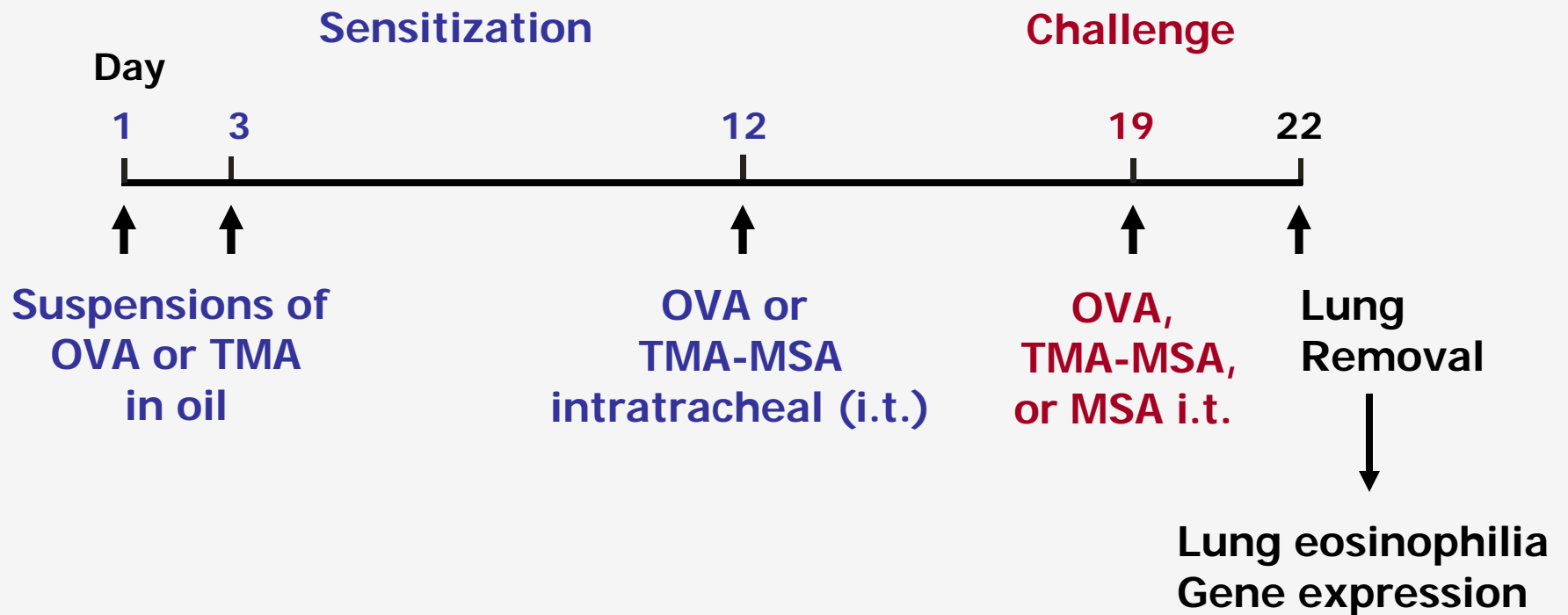
- **Commercially available**
  - **Affymetrix does GeneChip design, quality control and annotation**
  - **Murine Genome Chips**
    - **MG\_U74A v2 + MG\_U74Bv2 + MG\_U74Cv2**
    - **MG\_430 2.0**
- **University of Minnesota Core Facility supports Affymetrix products**

# Dose and Dosing Issues

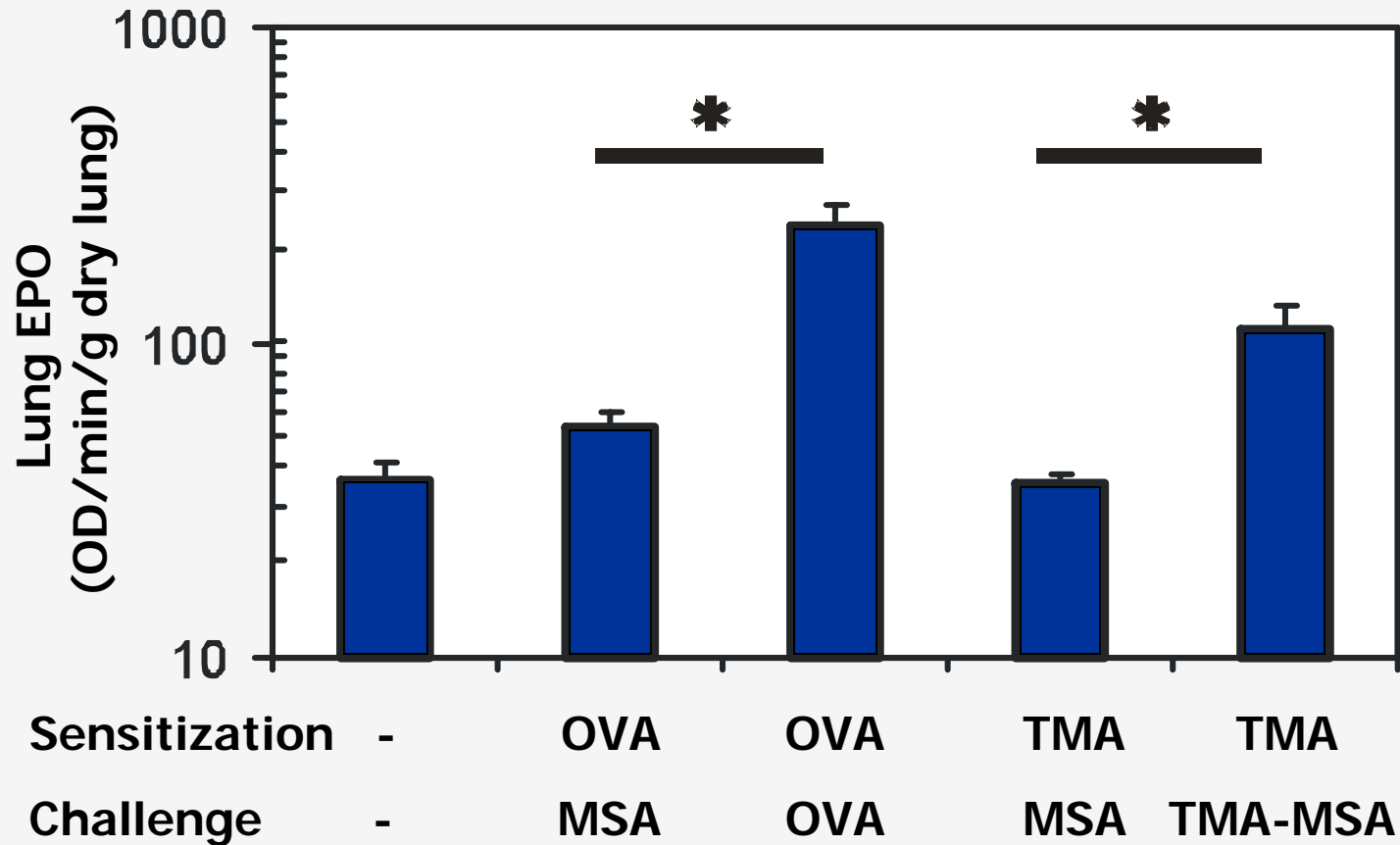
- **Sensitization and challenge regimen**
  - **Based on previous experience**
  - **Identical routes of exposure**
    - To insure that any differences in the biological response were due to allergen rather than sensitization/challenge regimen.
  - **Goal: Similar change in lung eosinophils in the effector phase**

# Experimental Design

## Sensitization and Challenge Regimen



## Lung Eosinophils



**Treatment Groups**

**OVAc**

**OVA**

**TMAc**

**TMA**

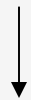
Comparison of OVA/OVAc vs TMA/TMAc: No difference

Lung Tissue (RNAlater or flash frozen in liquid N<sub>2</sub>)



RNeasy (Qiagen)

Total RNA (Quantitate by Spectrophotometer)



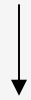
Superscript Choice (Invitrogen Life Technologies)

ds cDNA (Analyze by Gel Electrophoresis)



*In vitro* transcription with ENZO kit (Affymetrix)  
Clean up with RNeasy (Qiagen)

Biotin-Labeled cRNA (Analyze by Gel Electrophoresis)



Fragmentation buffer (Affymetrix)

Fragmented cRNA (Analyze by Gel Electrophoresis)



Mouse Chip U74Av2 → Data Analysis

**N=6-8 chips/treatment group; 1 animal per chip**

# How many chips?

- **What is the optimal 'n'?**
  - Power analysis in microarray experiments is complicated by the number of comparisons and the goal of detecting inter-related genes
  - Cui and Churchill (2003) recommend an 'n' of 6 or more to detect relevant biological changes
  - \$\$\$\$
- **To pool or not to pool**
  - \$\$\$\$
  - Quantity of tissue sample limits RNA yield
  - With pooling, other biological measures cannot be correlated with individual animal's genetic expression
  - Pooling may result in a larger SE than non-pooling i.e. you don't gain as much information as you might expect.
  - Still an area of active investigation

# Getting good RNA

- **RNase**

- Abundant in eosinophils and the allergic lung
- Inflamed lungs are more susceptible to RNA degradation than control lungs

- **Tissue processing**

- Lung lobes must be removed quickly (<1min) and immediately flash frozen or immersed in *RNAlater*
- Lung is minced immediately in *RNAlater*
  - Small pieces, tube rotation



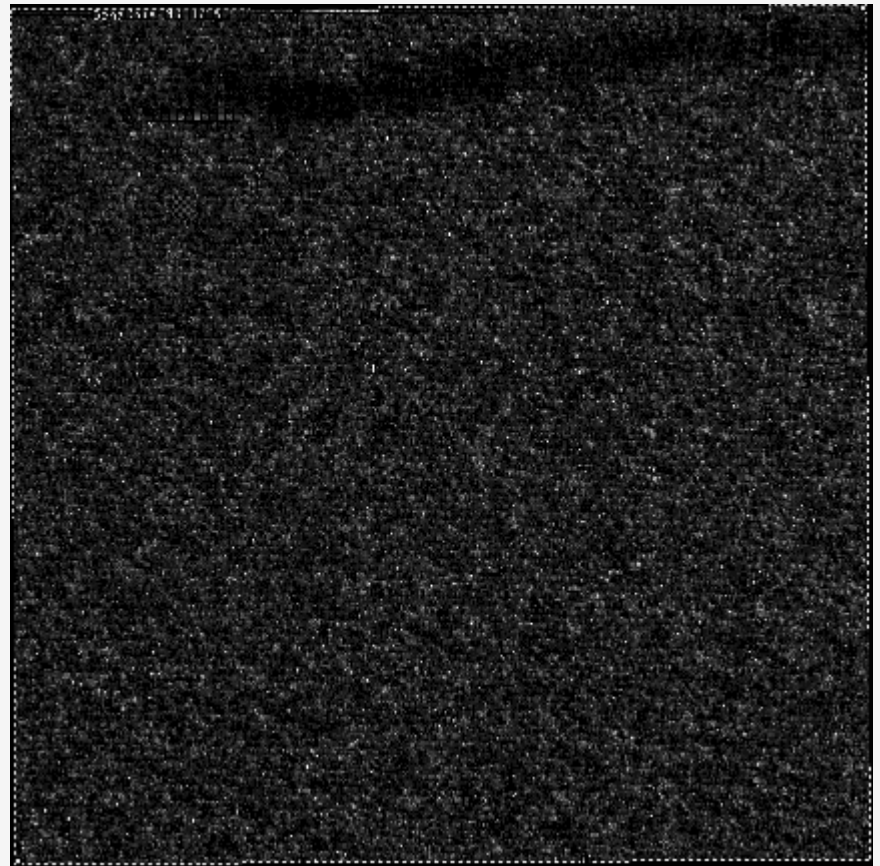
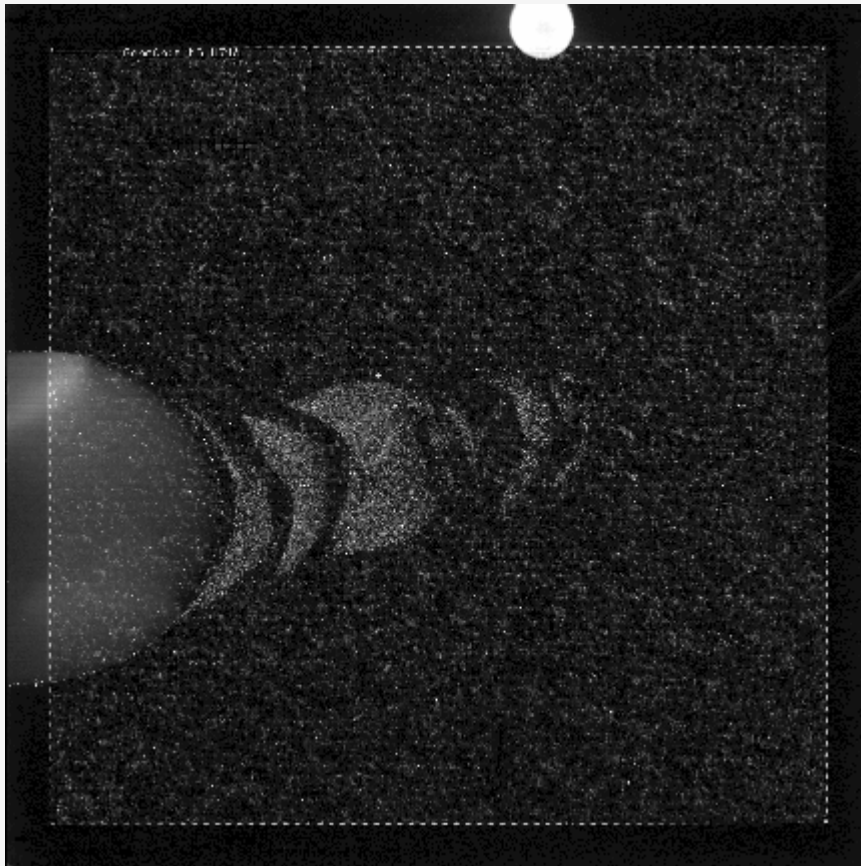
# Data Analysis

- **Determination of Intensities of Probe Sets**
- **Quality Control of GeneChip**
- **Normalization**
- **Determination of Differentially Expressed Genes**

# Data Analysis

- **Determination of Intensities of Probe Sets**
  - Affymetrix Microarray Suite 5.0 software
  - GCOS = Gene Chip Operating Software
    - To produce the .cel file
- **Quality Control of GeneChip**
  - Affymetrix Microarray Suite 5.0 or GCOS
    - Inspect image for gross flaws and abnormalities
    - Compare 5'/3' ratios, % present calls, background level and presence of spike controls in ChipReport to Affx standards
  - dChip software
    - Used to identify outliers
    - Newer statistical methods are superior
  - Bioconductor
    - Box plots
    - Plots of residuals and weights from robust model fitting

# Example of gross flaw or abnormality in AFFX images

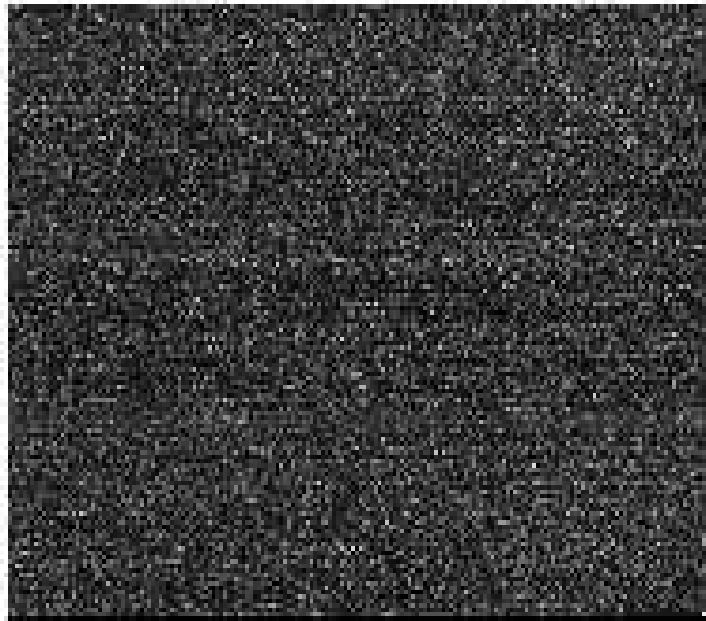


# Data Analysis

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# Outliers Identified by Bioconductor but not by Affx

DOD\_Ctrfoo\_356\_430\_2.CEL



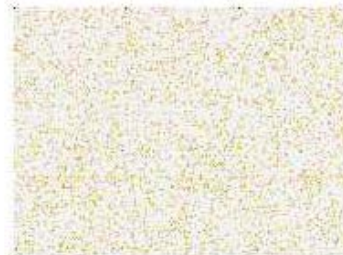
DOD\_Ctrfoo\_356\_430\_2.CEL



DOD\_Ctrfoo\_356\_430\_2.CEL



DOD\_Ctrfoo\_414\_430\_2.CEL



DOD\_Ctrfoo\_414\_430\_2.CEL



Weights

Residuals

# Data Analysis

- **Determination of Intensities of Probe Sets**
- **Quality Control of GeneChip**
- **Normalization**
- **Determination of Differentially Expressed Genes**

# Data Analysis

- **Normalization by the RMA Method**

- **Robust Multichip Analysis**
- **Accomplishes background correction, normalization and calculation of expression levels from chip intensities.**
- **Increases the power to detect effects for genes with low expression**
- **Uses only Perfect Match values from Affymetrix chips, not Mismatches**
- **Does not utilize present or absent calls from Affymetrix software**
- **Programs**
  - GeneTraffic
  - Bioconductor
  - Etc

# RMA Normalization

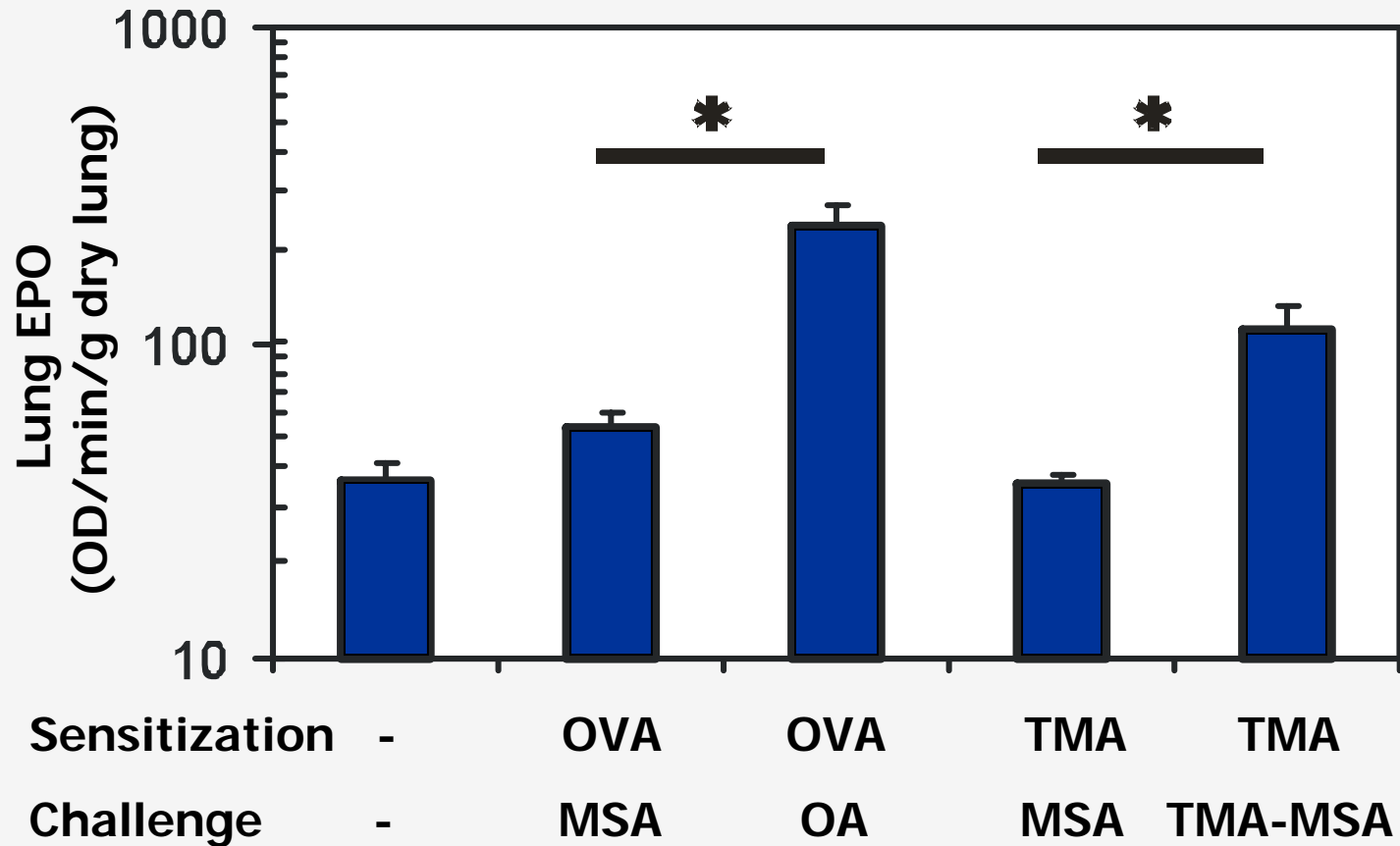
- **GeneTraffic**
  - Commercially available (Stratagene)
  - MIAME annotation
  - Central backup at University of Minnesota
  - User friendly
- **Bioconductor**
  - Free
  - Frequently updated and flexible
  - Only a statistician can love this program



# Data Analysis

- **Determination of Intensities of Probe Sets**
- **Quality Control of GeneChip**
- **Normalization**
- **Determination of Differentially Expressed Genes**

## Lung Eosinophils



**Treatment Groups**

**OVAc**

**OVA**

**TMAc**

**TMA**

Comparison of OVA/OVAc vs TMA/TMAc: No difference

# Statistical Analysis

- **Question 1: For each gene, is there any difference detected across the 4 treatment groups (OVAc, OVA, TMAc, TMA)?**
  - **Assuming unequal variances**
  - **F test using ANOVA in SAS to generate a p value**

# Statistical Analysis

- **Question 2: How do we guard against false positives with the large number of comparisons?**
  - **R software to generate q values for each probe set**
    - **ANOVA p values used to compute q values by the method of Storey & Tibshirani, 2003**
    - **q value is a type of False Discovery Rate**

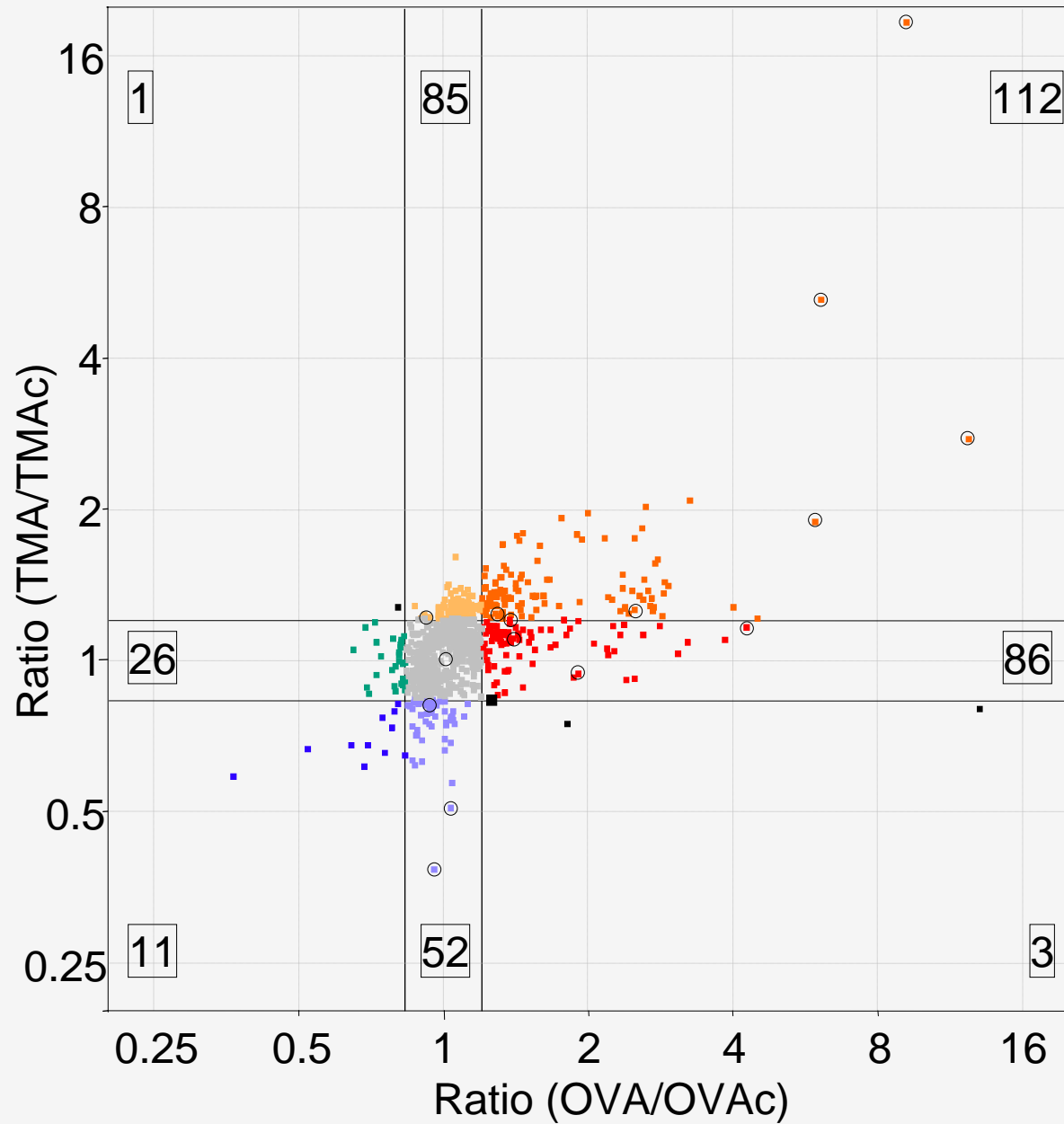
# Selection of differentially expressed candidate genes

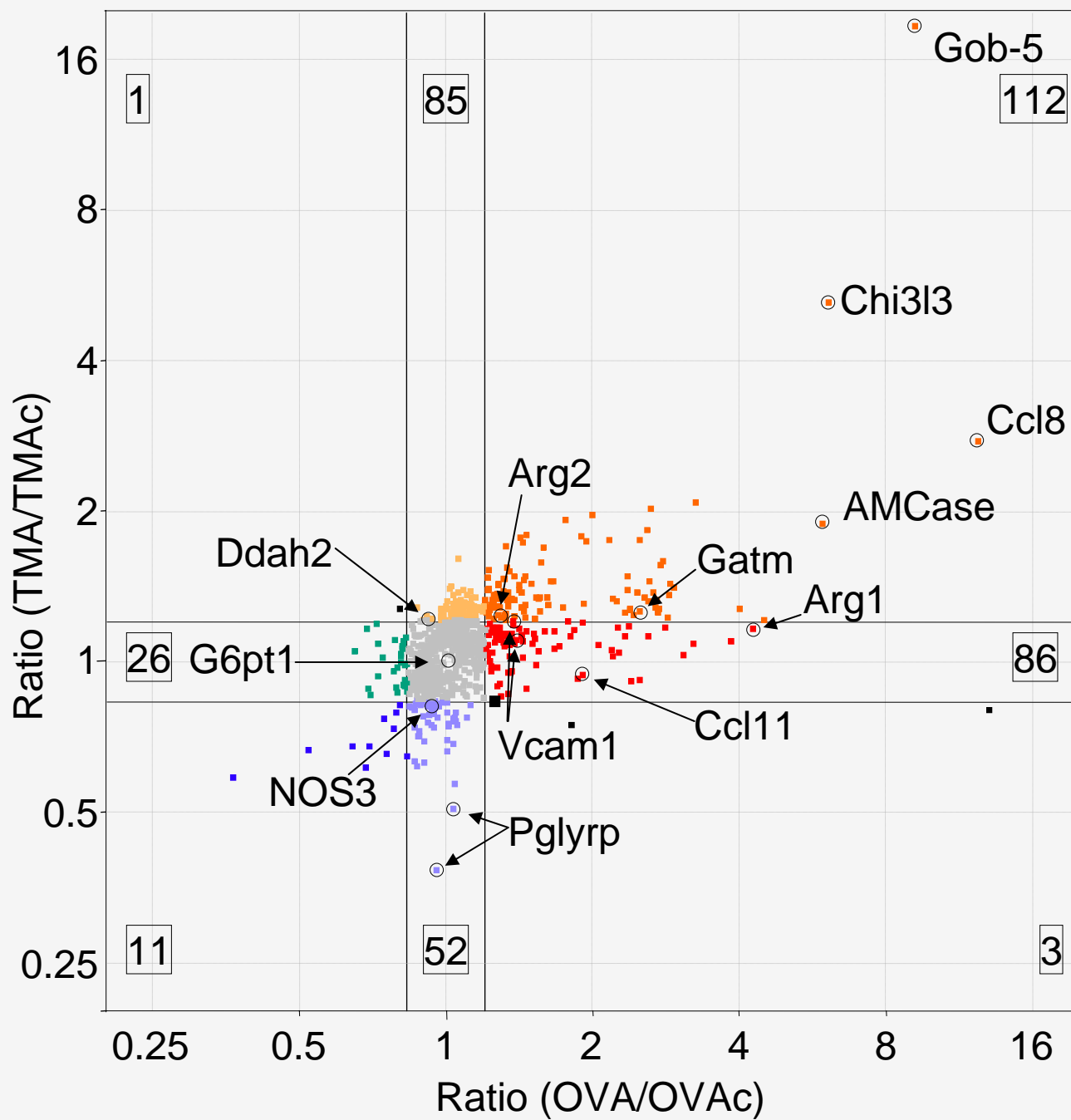
## ▪ Criteria

- **False Discovery Rate:  $q$  value  $< 0.1$** 
  - Accepting that 1 of 10 selected genes could be a false positive
  - Result: 855 probe sets
- **Magnitude of the change**
  - Ratio of gene expression for OVA/OVAc or TMA/TMAc is either  $> 1.2$  or  $< 1/1.2$
- **391 probe sets satisfy both criteria**

855 probe sets with  $q < 0.1$

391 probe sets with a ratio  $> 1.2$  or  $< 1/1.2$





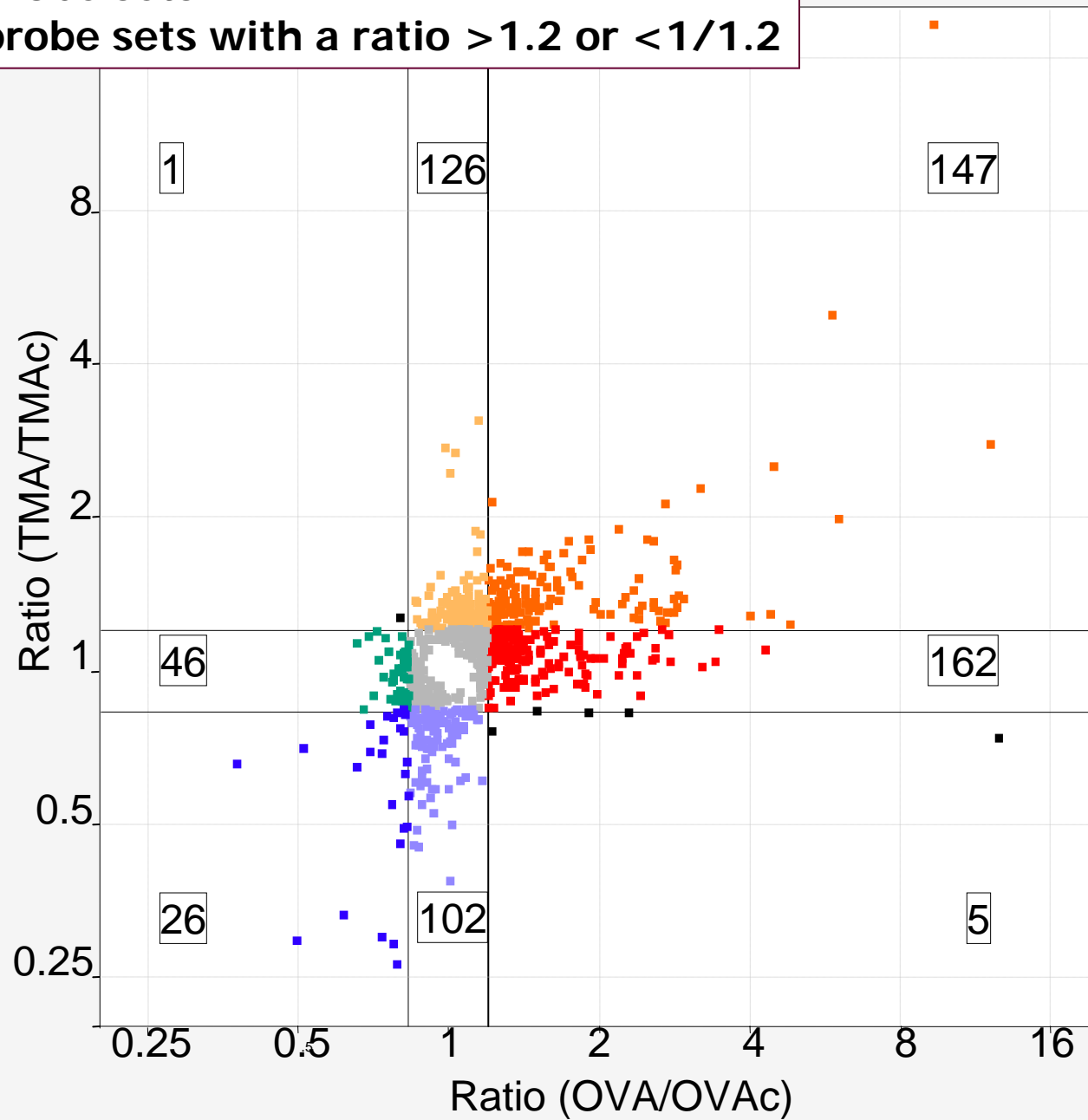
# Improved Statistical Analysis Using Empirical Bayes

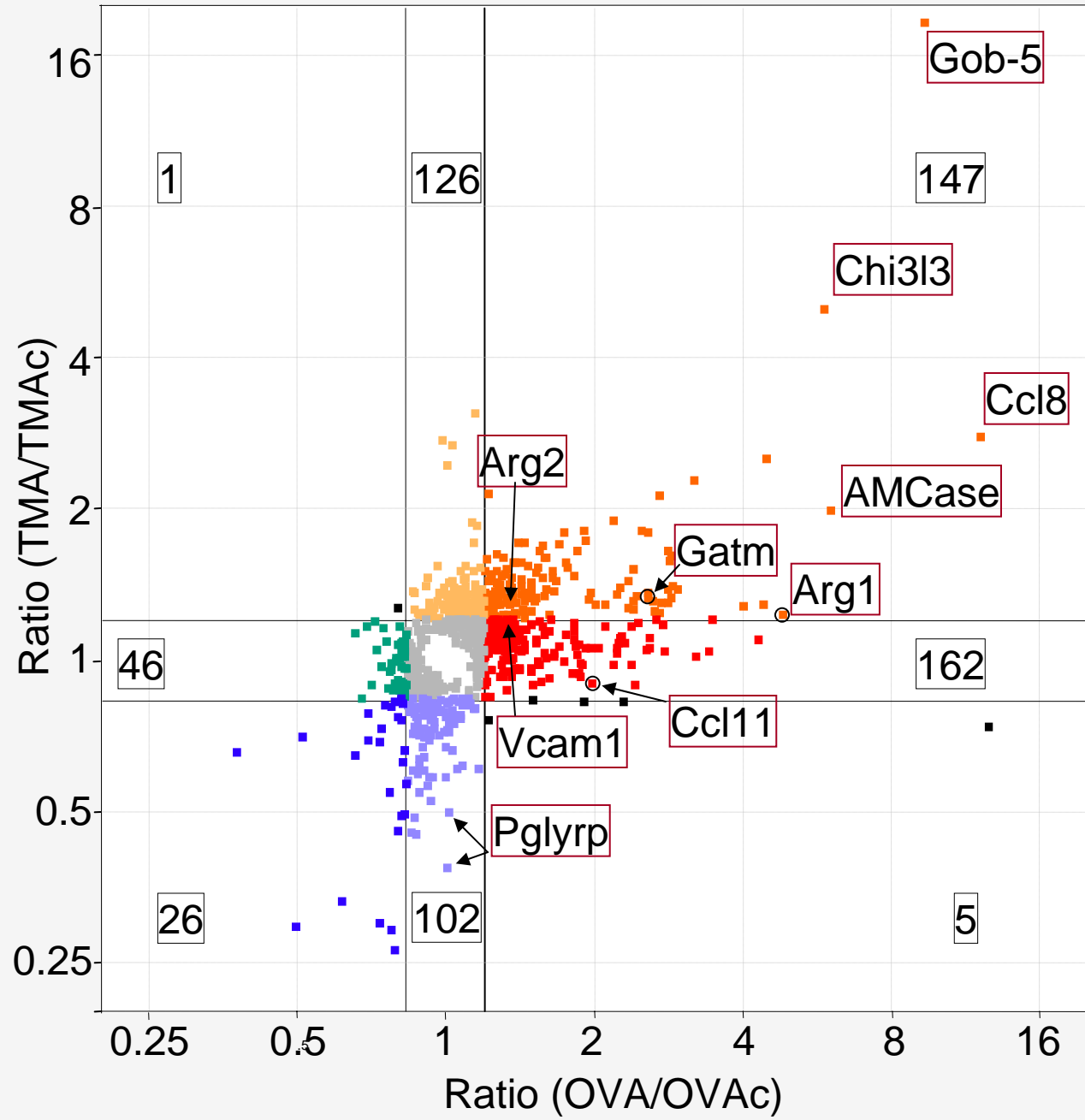
- **Question 1: For each gene, is there any difference detected across the 4 treatment groups (OVAc, OVA, TMAc, TMA)?**
  - **Moderated F statistic using a similar cutoff as in the previous analysis for purposes of comparison.**
  - **Moderated F statistic guards against small changes being significant because of misleadingly small variances**



843 probe sets

615 probe sets with a ratio  $> 1.2$  or  $< 1/1.2$

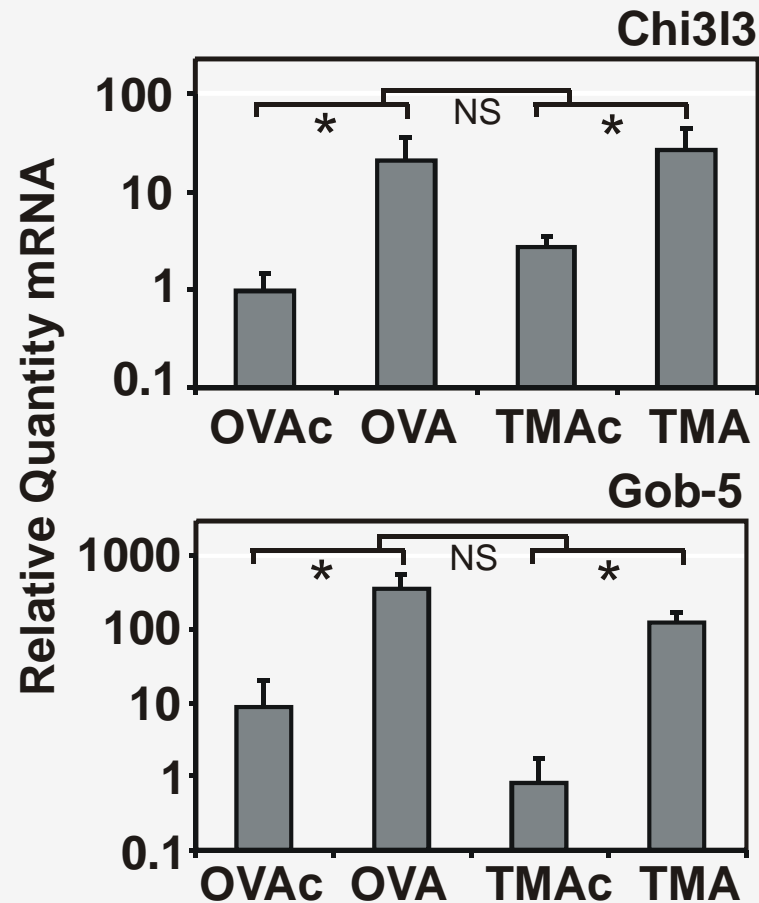




# Confirmation by qRT-PCR

Genes increased with both allergens

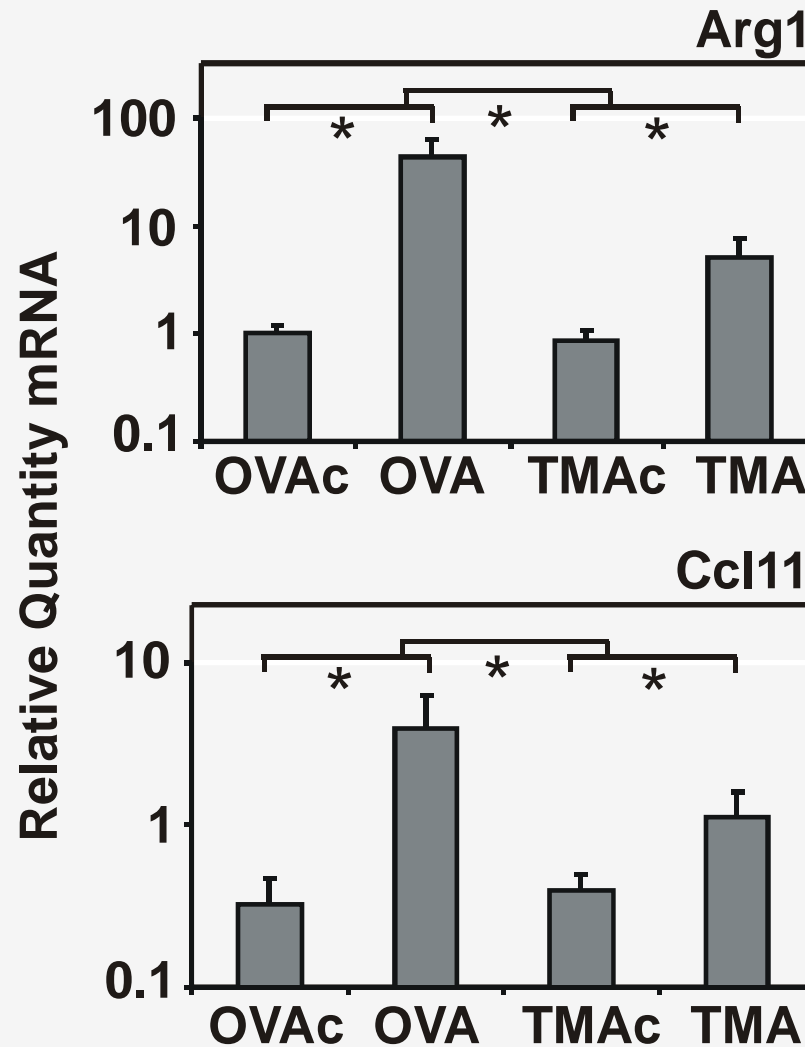
**Gob5 and Chi3I3**



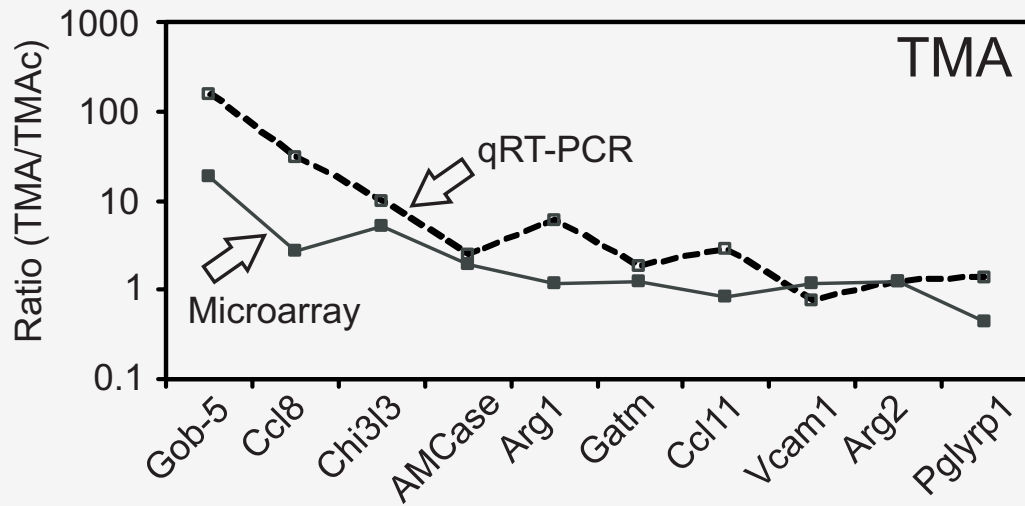
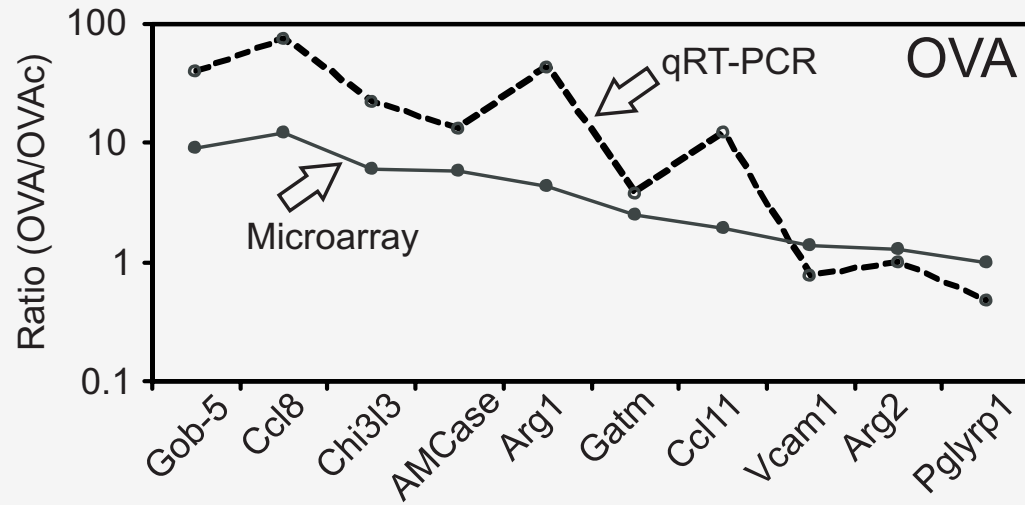
# Confirmation by qRT-PCR

Genes increased more with OVA than TMA

Arg1 and Ccl11



# Confirmation by qRT PCR

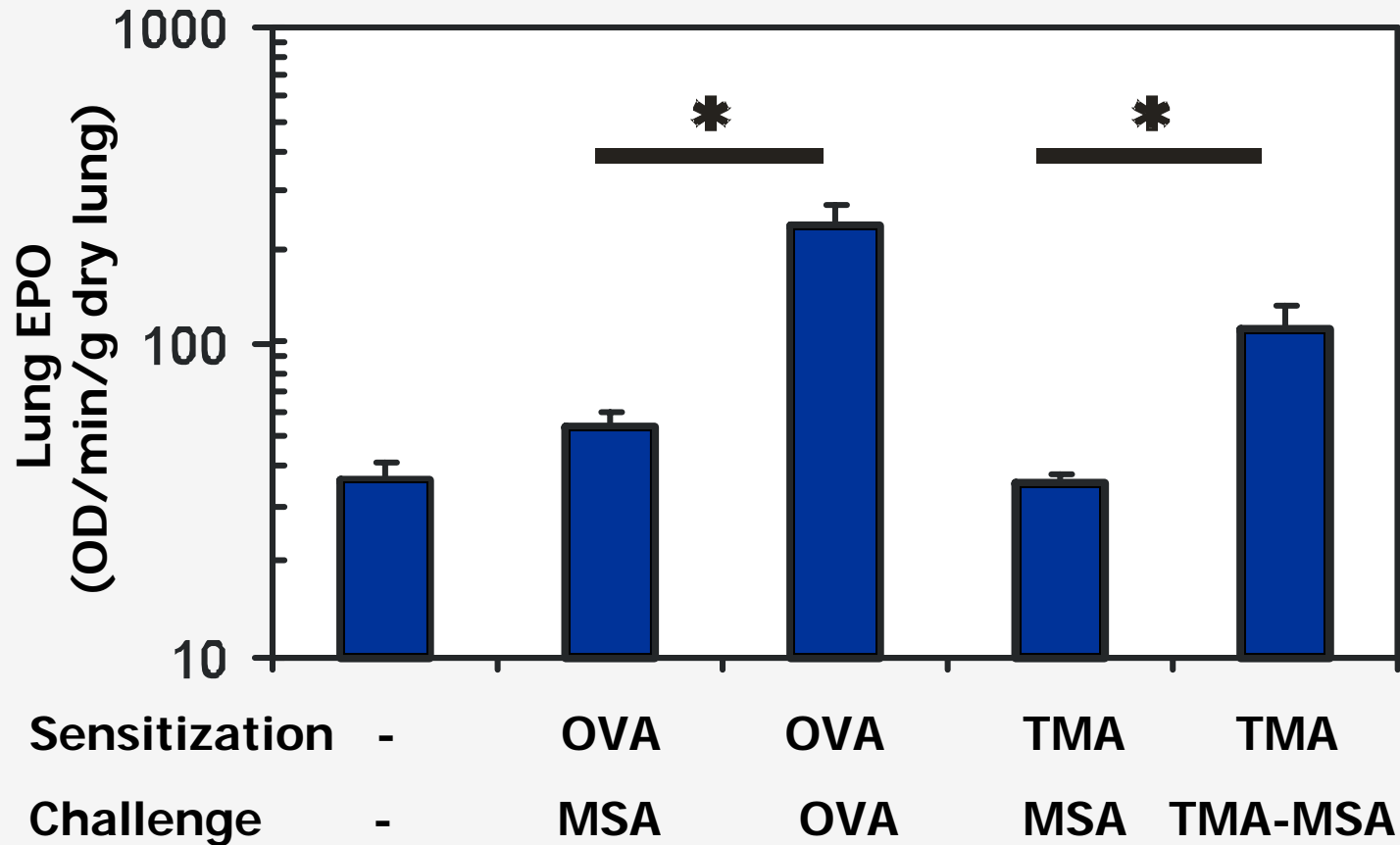


# Specific Aim

**Identify differentially expressed transcripts in the lungs of mice sensitized and challenged with either ovalbumin (OVA) or trimellitic anhydride (TMA)**

**Unique patterns of gene expression with different allergens suggest unique effector mechanisms or reflect heterogeneity of asthma symptoms.**

## Lung Eosinophils



**Treatment Groups**

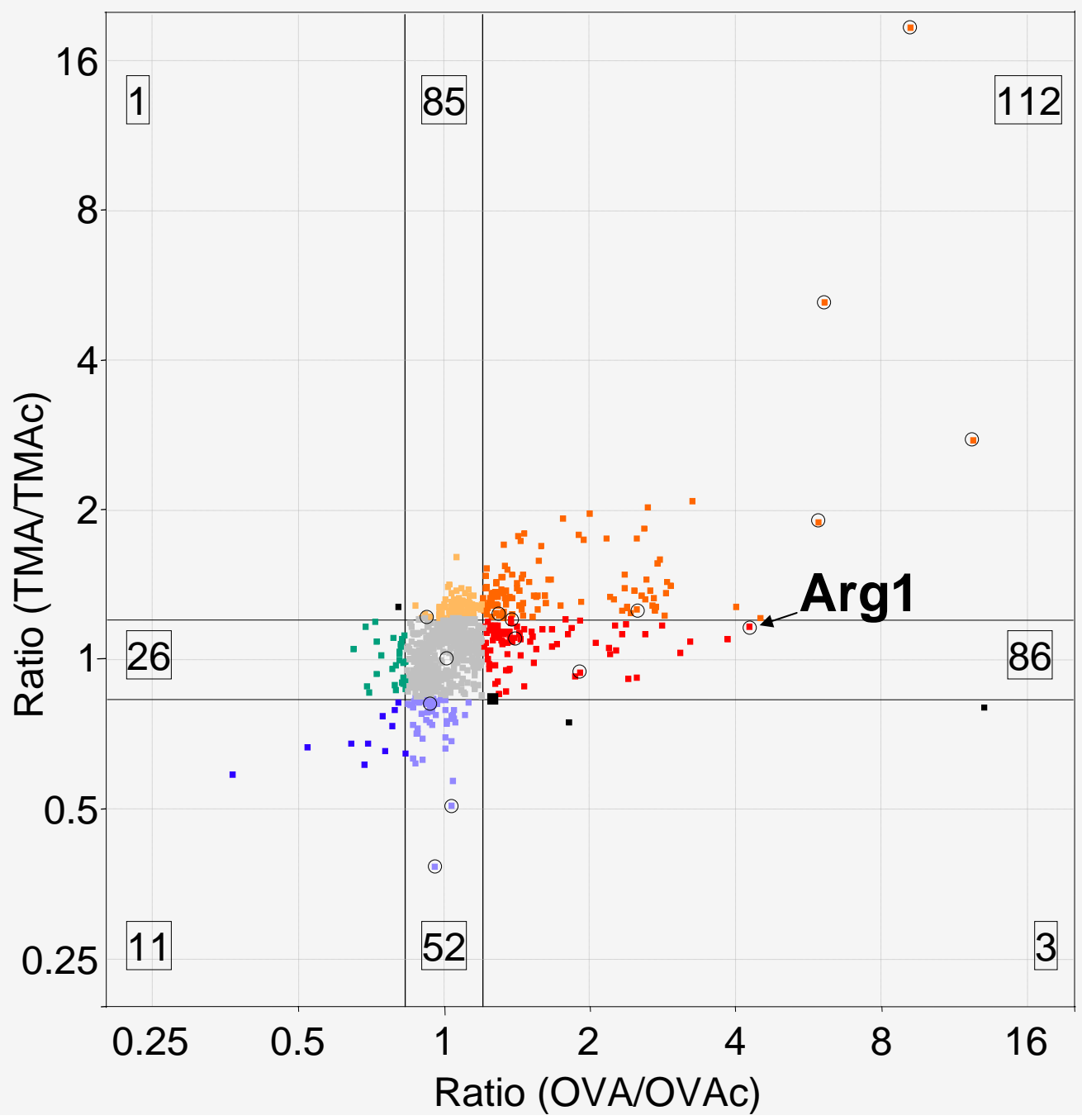
**OVAc**

**OVA**

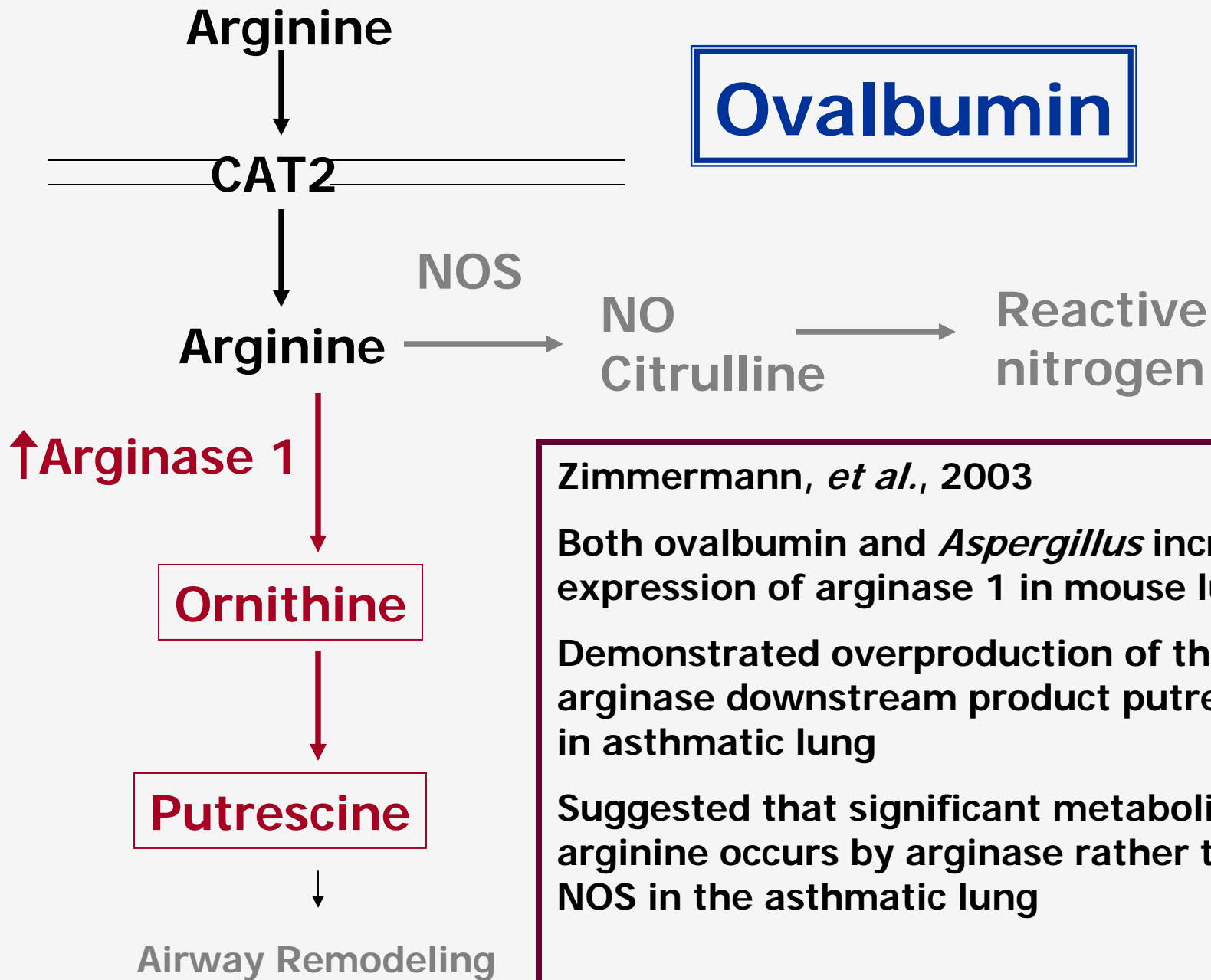
**TMAc**

**TMA**

Comparison of OVA/OVAc vs TMA/TMAc: No difference







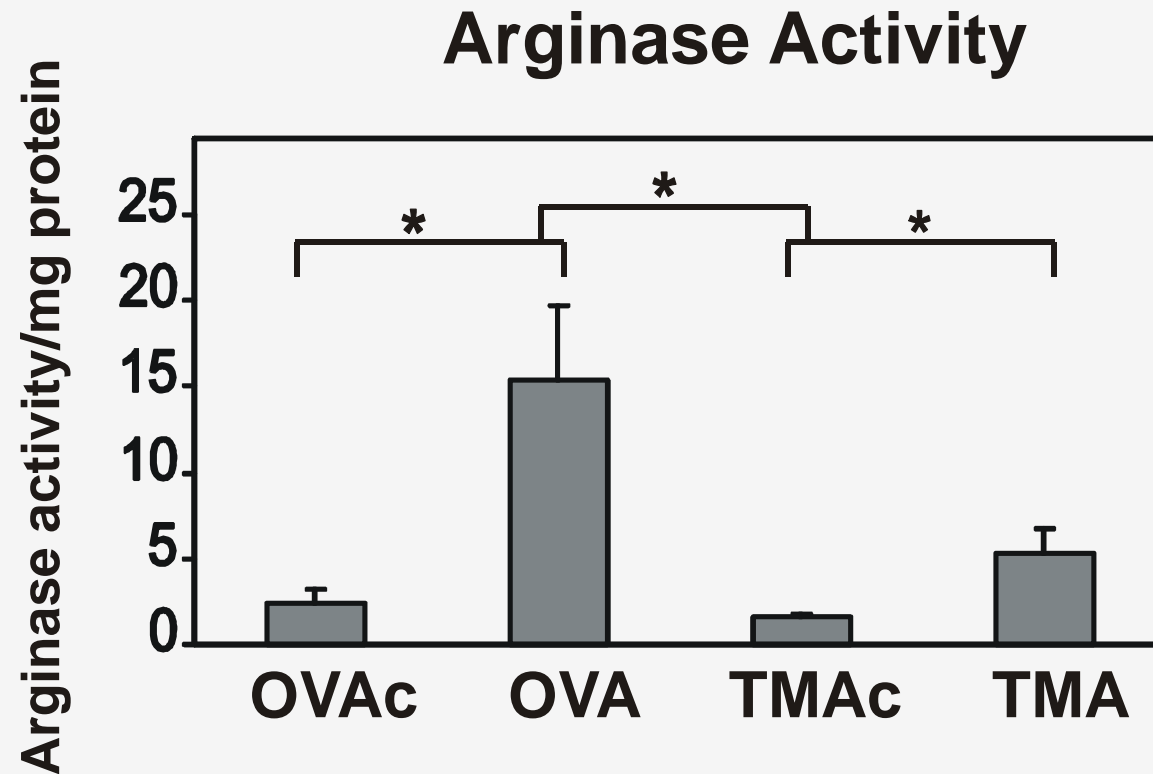
Zimmermann, *et al.*, 2003

Both ovalbumin and *Aspergillus* increased expression of arginase 1 in mouse lung

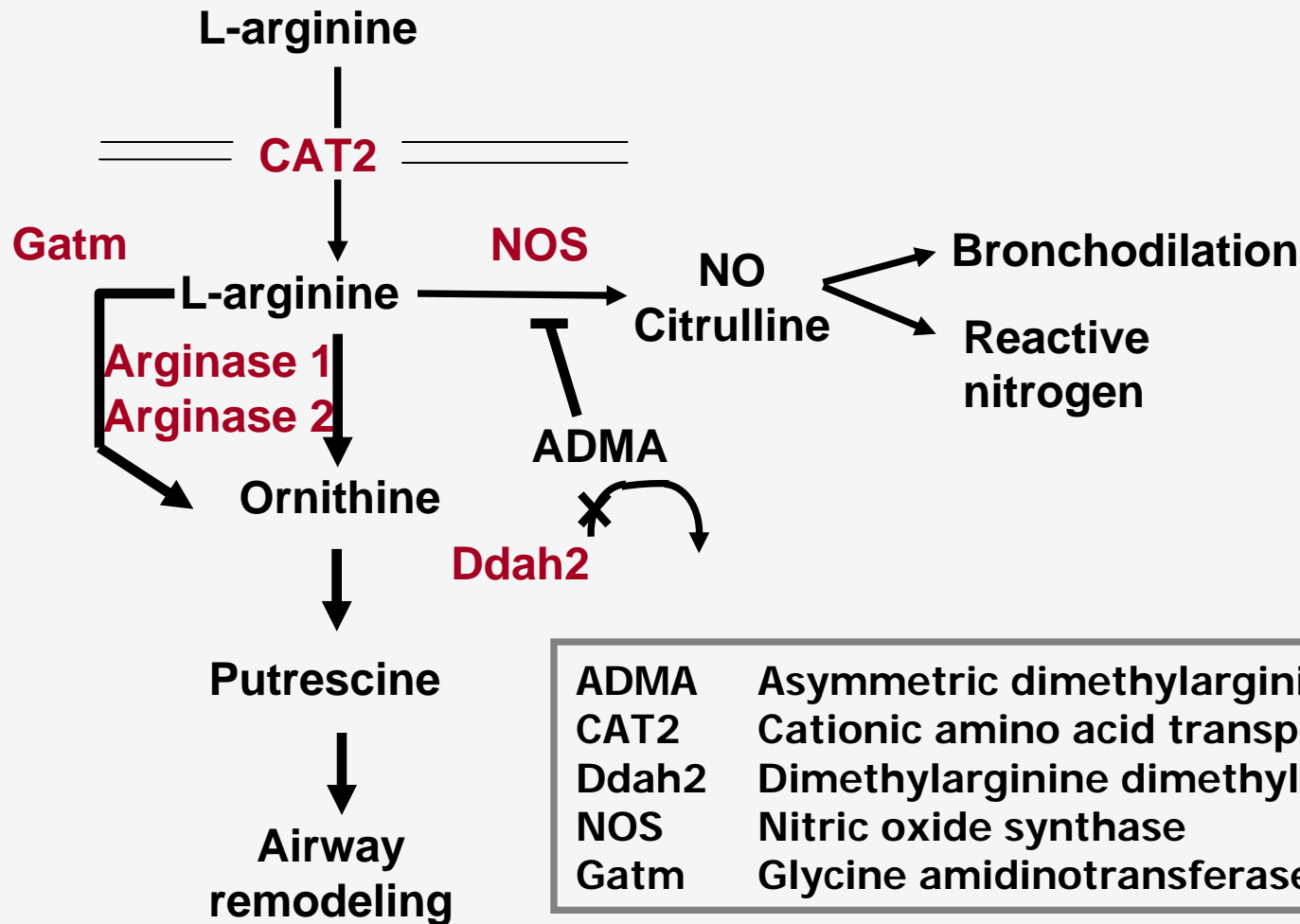
Demonstrated overproduction of the arginase downstream product putrescine in asthmatic lung

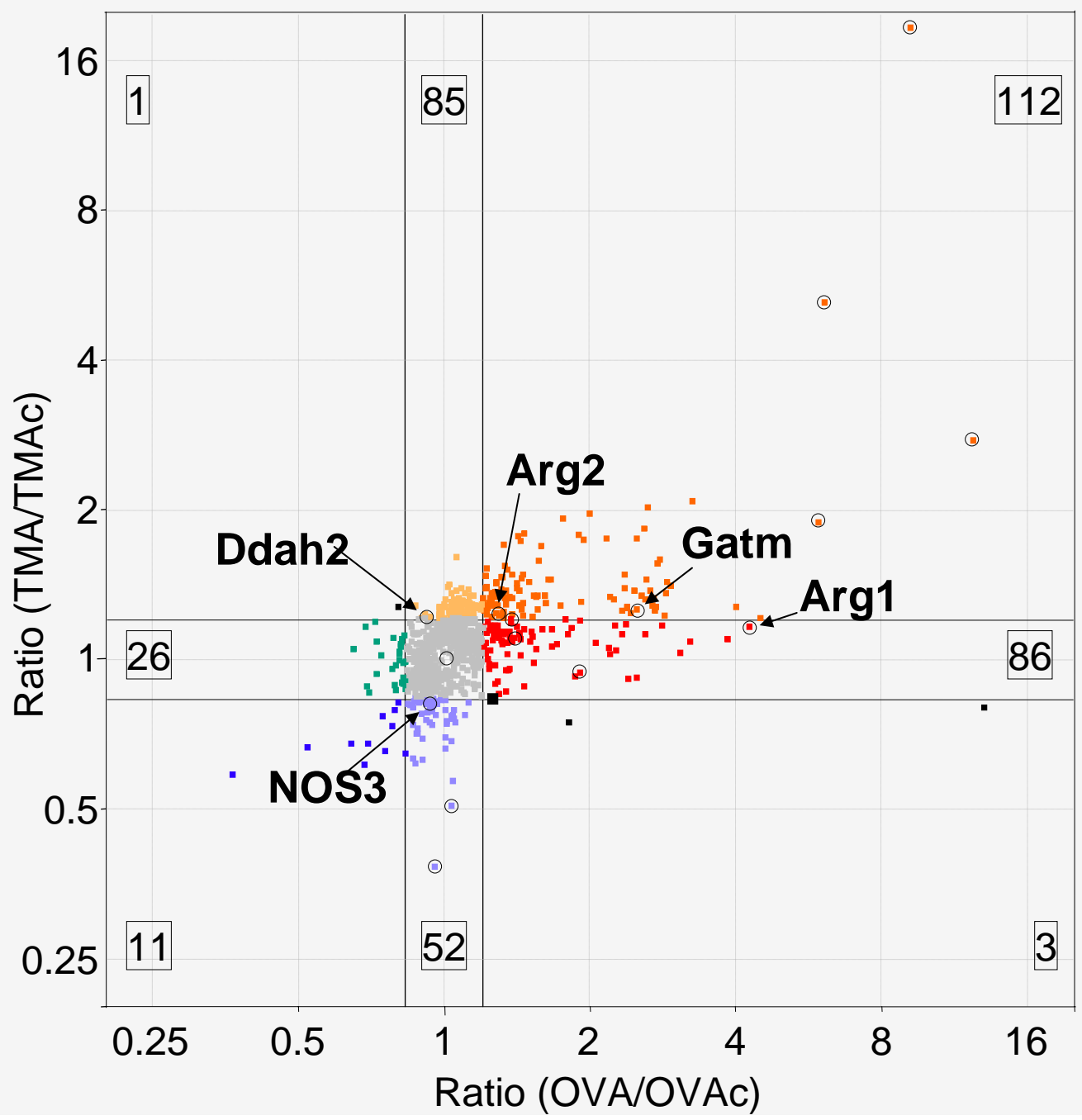
Suggested that significant metabolism of arginine occurs by arginase rather than by NOS in the asthmatic lung

# Arginase Activity in Lung



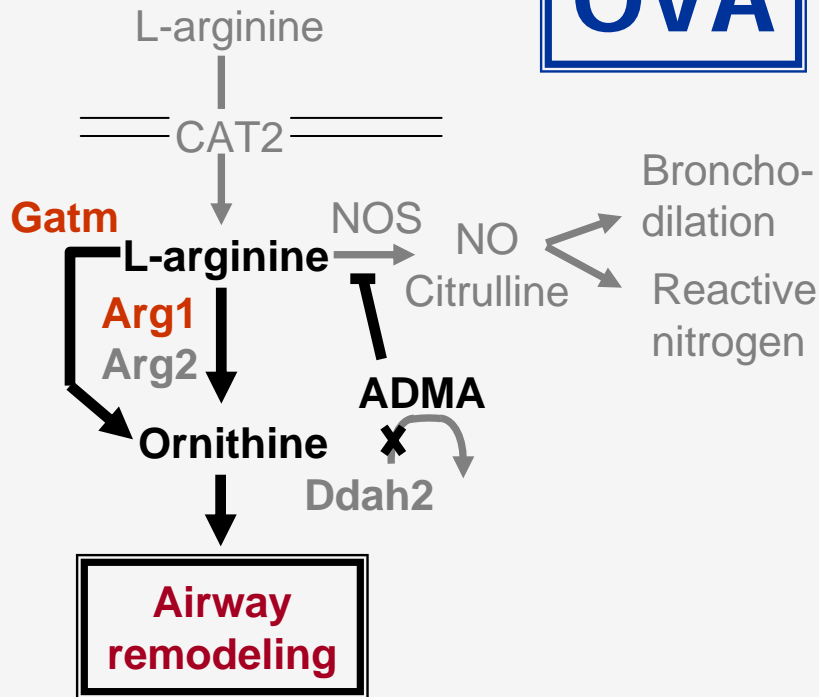
# Arginine Metabolism



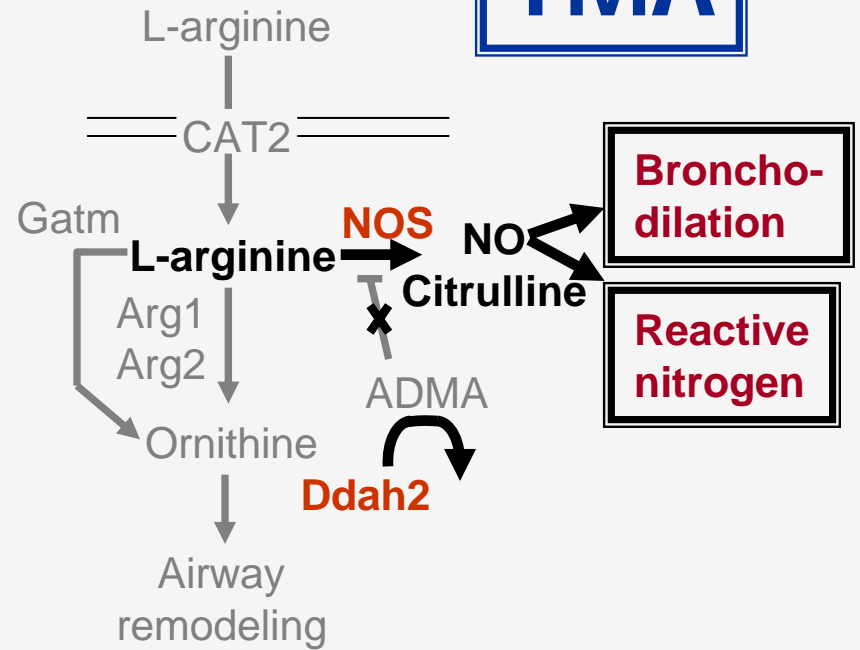


# Hypothetical Model of Mechanistic Differences

**OVA**



**TMA**



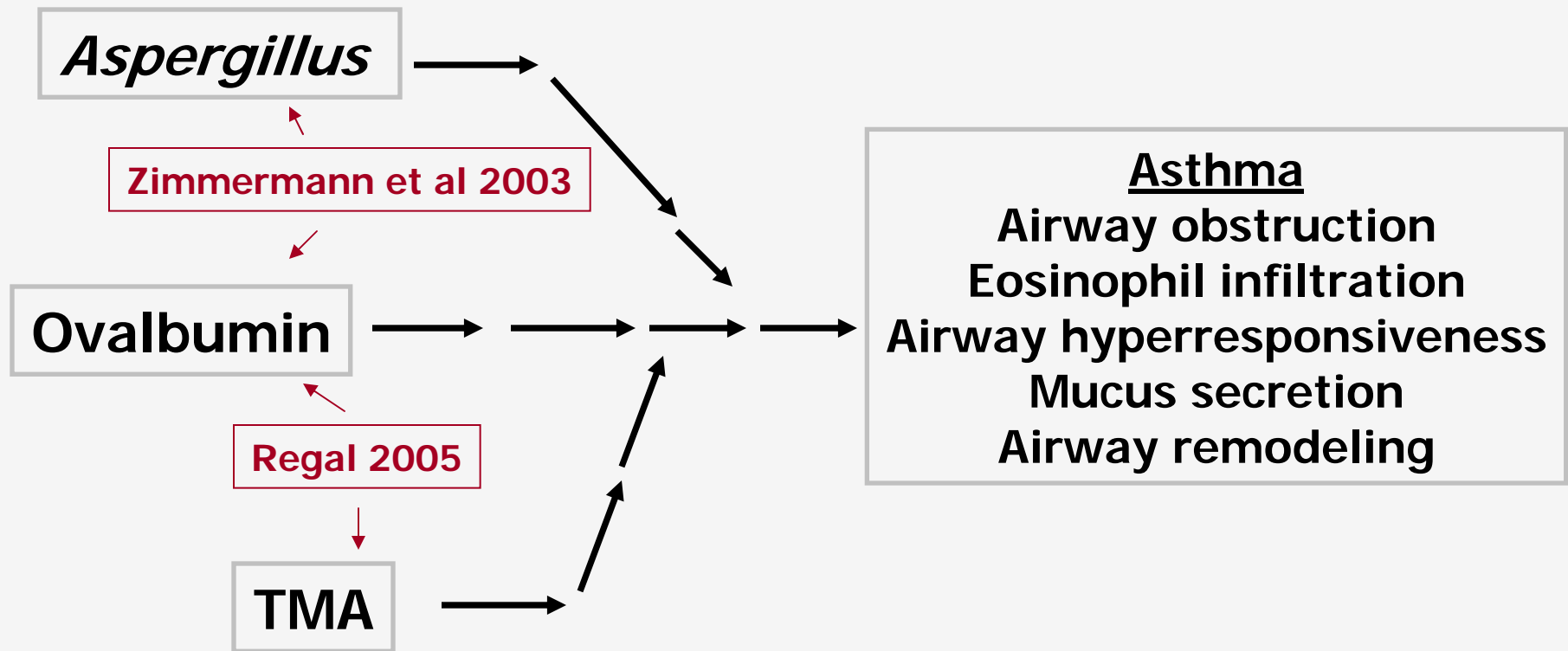
# Summary of OVA/TMA differences

- **Microarray analysis**
  - Differences in Arginase 1, Gatm and Ddah2 gene expression
- **qRT PCR analysis**
  - Confirms relative changes seen on microarray analysis for Arginase and Gatm
- **Measurement of Arginase enzyme activity**
  - Greater increase in OVA than TMA induced asthma models, consistent with differences in message

# Conclusion

- Pathways of arginine metabolism and the importance of nitric oxide in asthmatic inflammation may differ in OVA and TMA induced asthma.
- Differences in gene expression may reflect
  - different pathways to the asthma symptoms
  - different profile or subset of asthma symptoms *i.e.*, asthma heterogeneity

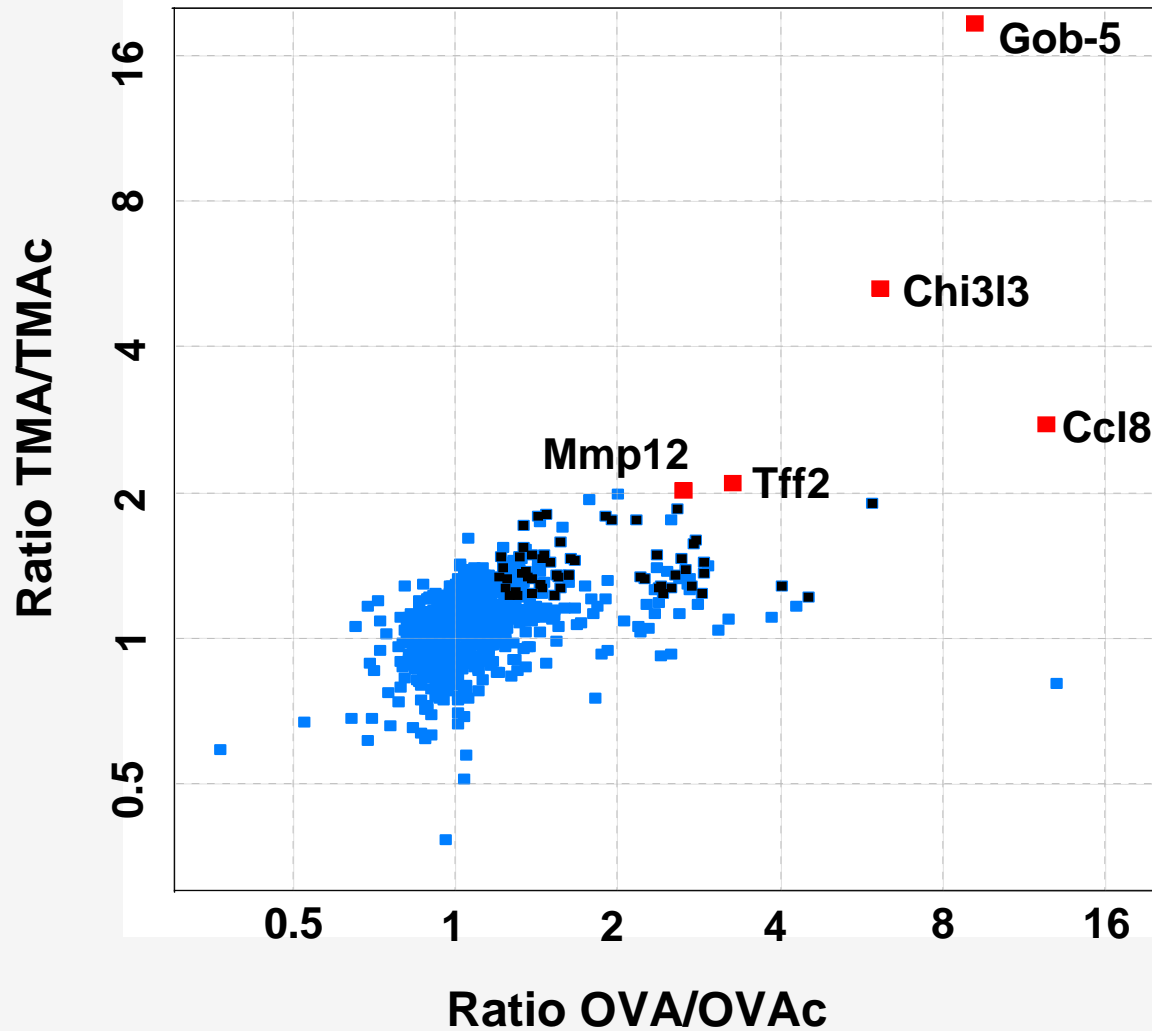
# Hypothetical Effector Pathways for Asthma



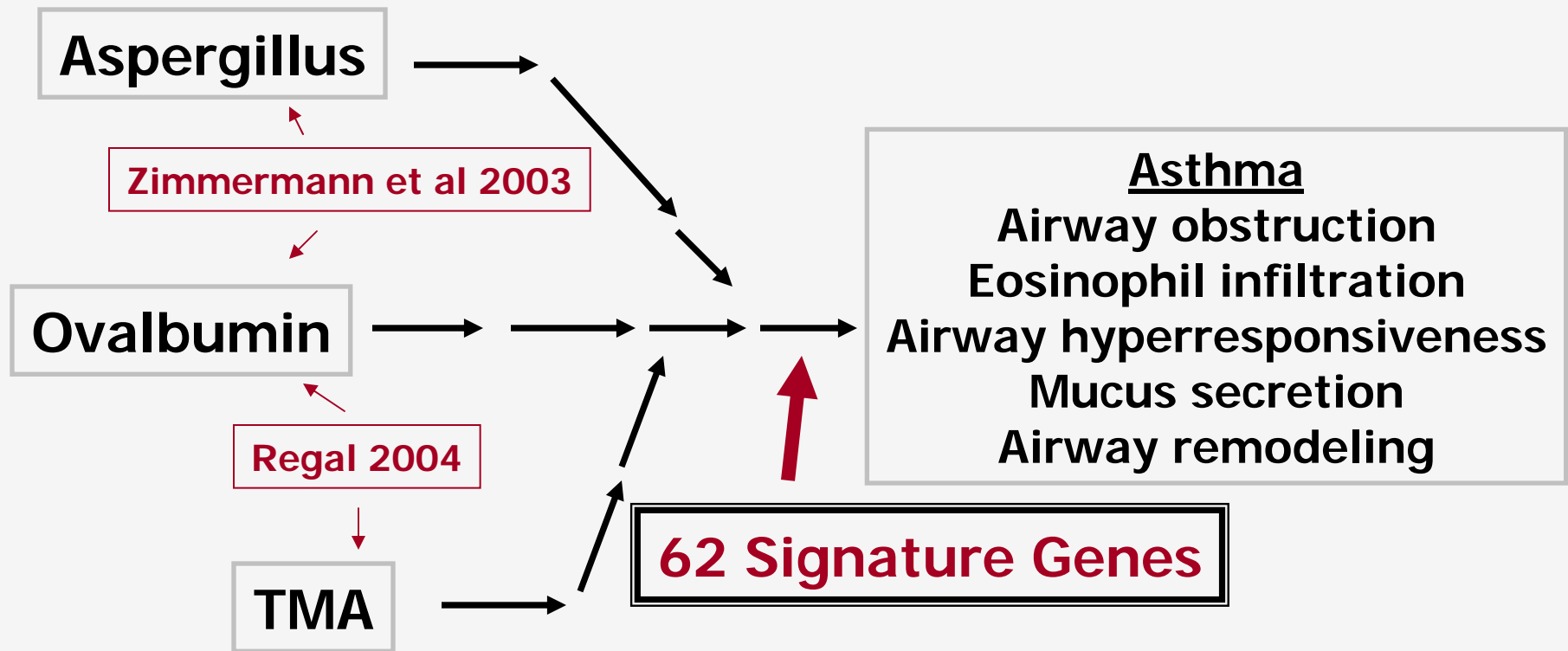


# Allergic Airway Inflammation Signature Genes

62 genes up-regulated in OVA-, TMA- and *Aspergillus*-Induced Allergic Airway Inflammation



# Hypothetical Effector Pathways



# Conclusion

- **OVA and TMA evoke unique patterns of gene expression in the lung**
- **Signature genes for allergic airway inflammation may define the events common to multiple antigens in the effector phase of asthma**

# **Is array technology useful for screening to predict respiratory hypersensitivity?**

- **Events common to the induction phase of asthma are more practical as screening techniques**
- **Techniques would need extensive validation with a variety of respiratory allergens and negative controls**

# General implications for Immunotoxicologists

- **Microarray techniques**
  - **Well suited for looking for novel differences in mechanisms of effector pathways**
    - **Implications for differential therapy of asthma depending on allergen**
  - **Attention to experimental design and statistical analyses are critical**