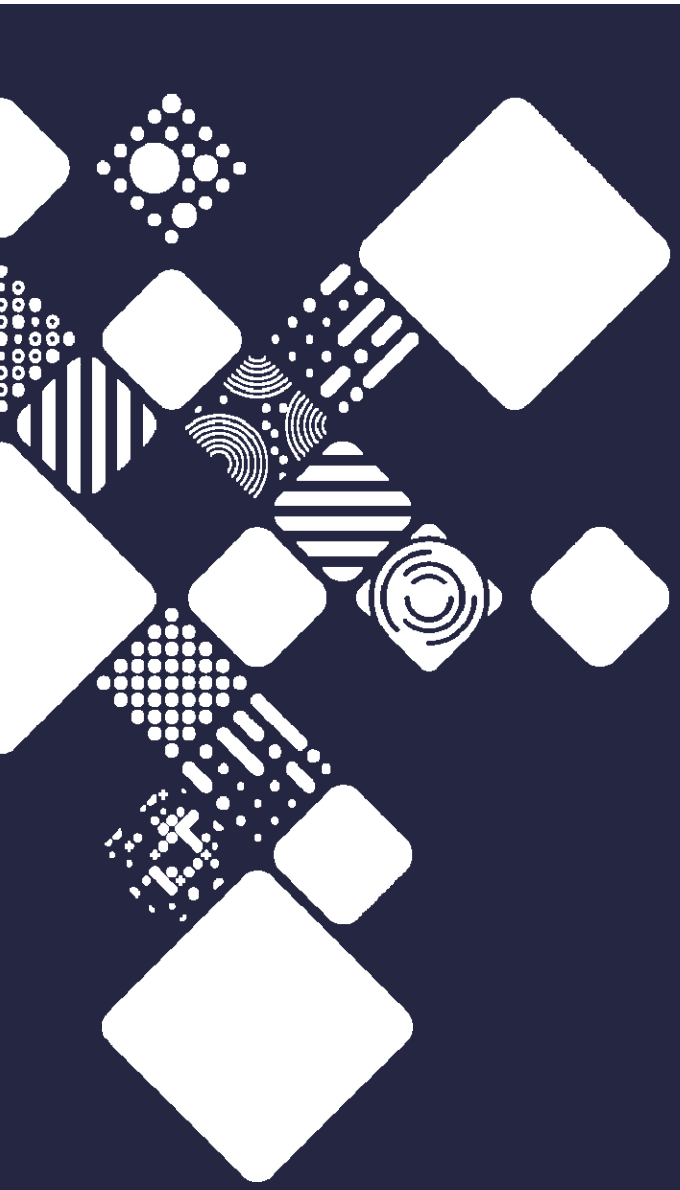


Cryptic mitochondrial diversity drives the development of *Plasmodium falciparum* drug resistance

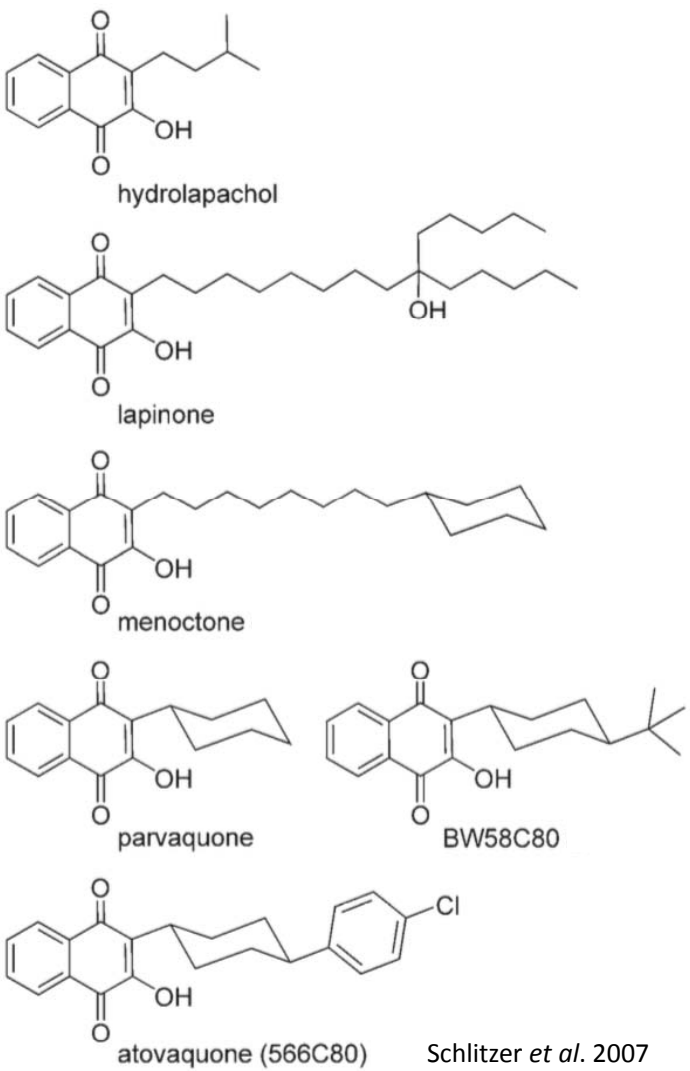
Sasha V. Siegel, Ph.D.
Wellcome Trust Sanger Institute
Rayner Lab
December 12, 2018

Historical rapid resistance development to monotherapy

Antimalarial drug	Year of introduction	1st case of resistance	
Quinine	1632	1910	
Chloroquine	1945	1957	*12 years*
Proguanil	1948	1949	*1 year*
SP	1967	1967	*<1 year*
Mefloquine	1977	1982	*5 years*
Atovaquone	1991	1991	*<1 year*

Atovaquone

- Hydroxynapthoquinone, ubiquinone analog, binds cytochrome *b* Q_o site
- Collapses mitochondrial membrane potential
- Broad spectrum antiprotozoal activity
- Efficacy in blood and liver stages

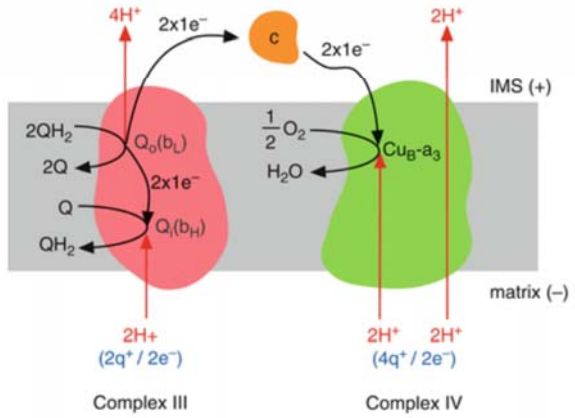


Schlitzer *et al*. 2007

- ~67% cure rate in monotherapy
- Synergistic with proguanil partner drug (Malarone)
- Slow onset of action
- Used for treatment and causal prophylaxis

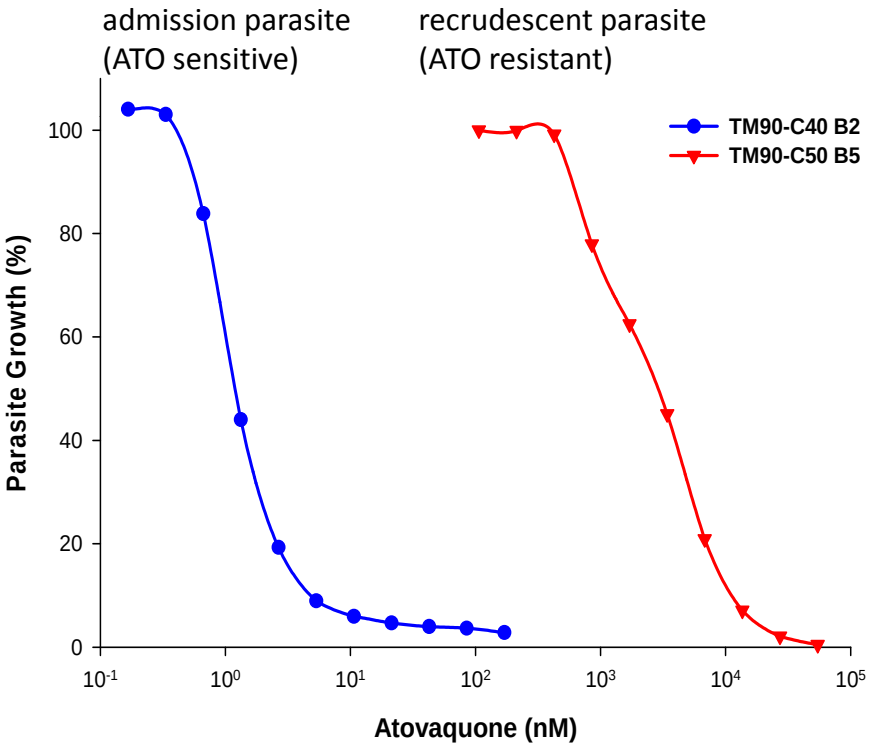
mETC targets: cytochrome bc_1 complex

- bc_1 catalyzes electron transfer: ubiquinol to cyt *c*
- Electron transfer coupled to translocation of protons across the inner membrane
  - results in electrochemical gradient for ATP production by ATP synthase (Complex V)
- ATQ inhibits ubiquinone regeneration by mitochondrial cyt. bc_1 (Complex III)
  - collapse of mitochondrial membrane potential
  - Prevents pyrimidine production, stops DNA synthesis



Biagini *et al.* 2014

Atovaquone resistance



Looareesuwan *et al*. 1996

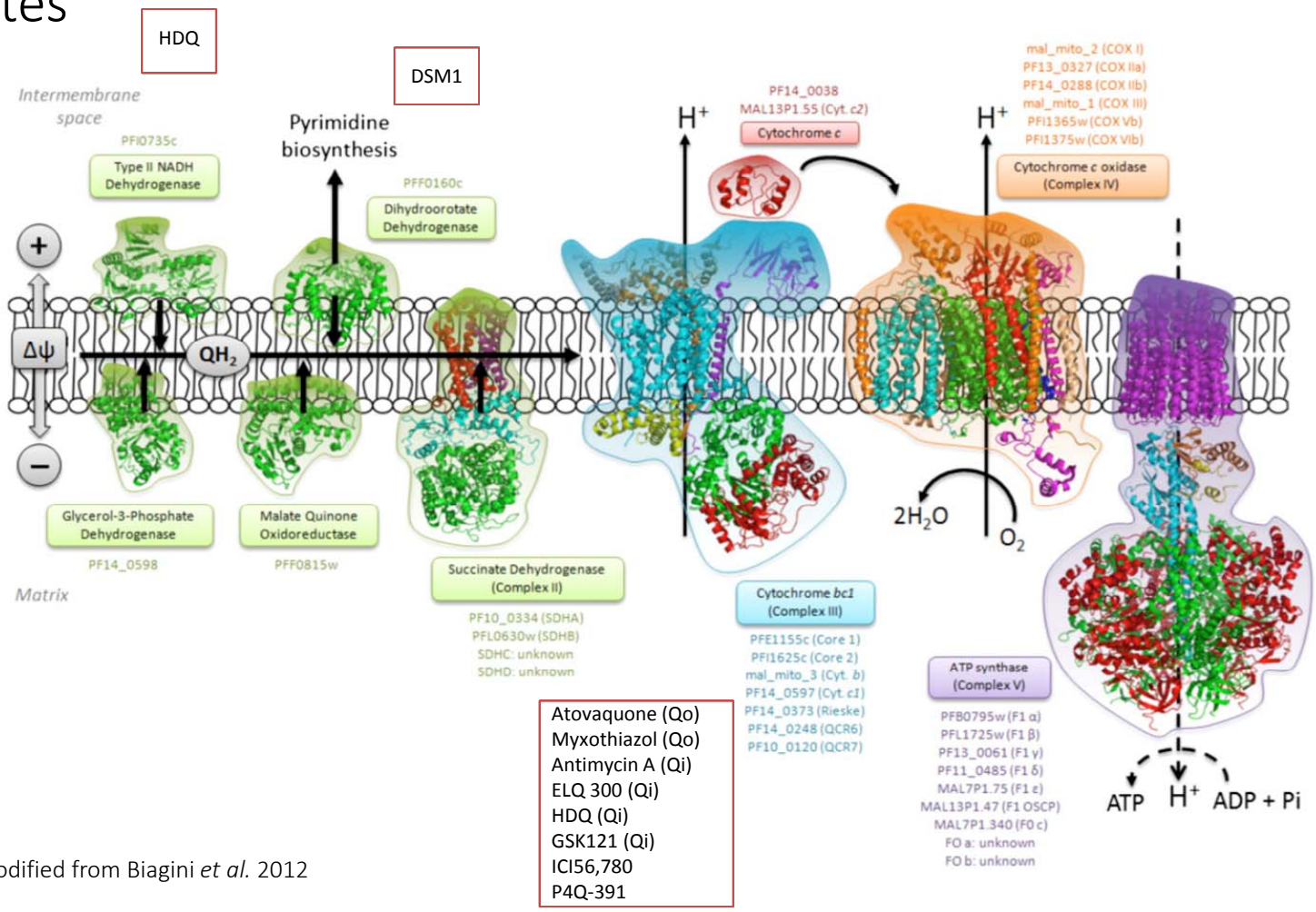
- Mutations in cytochrome *b* at Y268: S, N, C substitutions
- Some ATO resistant parasites have no mutation
- Sporadic treatment failures with Malarone
- Parasites have varied response to ATO (5-10,000 fold)

Parasite history for paired admission and recrudescence *Pf* isolates from Thailand Phase II studies

Treatment Regimen	Patient No.	Admission/Recrudescent	Isolate	Cyt. *b* mutation
ATOV 750 mg q8h x 4	2	A R	C2A C2B	-- Y268S
	6	A R	C6A C6B	-- --
ATOV 750 mg q8h x 21	29	A R	C40 C50	-- Y268S
	32	R	C32B	Y268N
ATOV 1000 mg plus PYR 25 mg q24h x 3	210	A R	C1028 C1086	-- Y268S
	207	A R	C1051 C1090	-- Y268N
	206	R	C1088	Y268S

Looareesuwan *et al*. 1996

Pf mitochondrial electron transport drug targets: Structure activity study of patient isolates



Modified from Biagini *et al.* 2012

Structure-activity study: extreme resistance seen in *Pf* suggests additional mechanisms of resistance to mtETC inhibitors

Low, moderate, and extreme resistance to mitochondrial electron chain inhibitors in *P. falciparum.* (EC_{50}s, nM)

			Complex III						
	NDH2	DHODH	Qo site (cyt *b*)		Qi site (cyt *b*)		unknown		
	HDQ	DSM-1	ATOV	MYX	ANT	ELQ 300	GSK121	ICI56,780	P4Q-391
W2	55.0	52.4	0.41	10.8	165.5	2.72	3.39	0.03	11.1
TM90-C6B	ND	24.5	109	7.45	125	7.67	36.4	0.50	33.0
TM90-C2A	14.8	79.4	3.09	153.7	72.0	0.69	20.2	0.04	1.92
TM90-C2B	146.5	57.4	5288	428.4	151.6	4.61	77.5	14.3	55.5
TM90-C40B2	829.6	47.2	1.53	45.1	72.9	4.02	7.84	0.04	22.1
TM90-C50B5	ND	ND	3940	3014	ND	6.45	122	13.4	32.4
TM92-C1086	179.6	305.3	44308	4105	>18230	16341	2473	882.2	14159
TM90-C1088	252.2	540.5	29095	3989	>18230	21000	3470	219.3	>20200

DSM-1: triazolopyrimidine; DHODH inhibitor

ICI56,780: phenoxyethoxy-4(1H)-quinolone; complex III inhibitor

GSK932121A (GSK121): 4(1H)-pyridone; complex III inhibitor

P4Q-391: 4(1H)-quinolone; complex III inhibitor

HDQ: 1-hydroxy-2-dodecyl-4(1H)quinolone; cyt *b* Qi site, NDH2 inhibitor

Atovaquone (ATOV): 2-hydroxynapthoquinone; cyt *b* Qo site inhibitor

Myxothiazol (MYX): cyt *b* Qo site inhibitor

ICI56,780: phenoxyethoxy-4(1H)-quinolone; complex III inhibitor

Antimycin A (Ant A): cyt *b* Qi site inhibitor

ELQ 300: cyt *b* Qi site inhibitor

Atovaquone (ATOV) treatment failures:

WT cytochrome *b* = low level ATOV resistance

Y268S/N = resistance that varied significantly among isolates

Atovaquone resistance dogma

- Resistance develops following treatment *in vivo*
- Rapid *de novo* selection of cytochrome *b* mutants *in vitro*
- Atovaquone resistance frequency is used to compare new drugs in early drug discovery ($10^6 – 10^8$)

<u>Questions that challenge the dogma</u>

- If resistance is due to a single cyt *b* SNP, why do we observe a wide range of resistance in vitro (4 – 10,000 fold)?
- Why are some parasites from treatment failures WT for cytochrome *b*?
- Why do *in vitro* drug selections not yield the clinically relevant Y268 mutants?

Clinically relevant Y268 mutations are not selected *in vitro*

Parasite name	cyt. *b* mutation(s)	Source
3D7	M133I M1331 & P275T M133I and K272R M133I and G280D L283I & V284K	Korsinczky *et al.* 2000
3D7	M133V M133I M133I & L144S F267V	Bopp *et al.* 2013
K1	M133I M133I & G280D	Schwobel *et al.* 2003
AT200	M133I M133I & L271F	Schwobel *et al*. 2003

Does the parasite genetic background determine the atovaquone resistance genotype selected?

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Variations in frequencies of ATO resistance in *Pf* isolates

Table 1. Susceptibility patterns of some *P. falciparum* clones

Compound	Clones and origin				
	W2 (Indochina)	FCR3 ("The Gambia")	HB3 (Honduras)	3D7 ("Netherlands")	D6 (Sierra Leonne)
Chloroquine	R	R	S	S	S
Quinine	R	—	—	—	S
Pyrimethamine	R	S	R	S	S
Cycloguanil	R	R	S	S	S
Sulfadoxine	R	S	S	R	S
5-Fluoroorotate	S	S	S	S	S
Atovaquone	S	S	S	S	S

Table 3. Frequency of resistance to atovaquone

Initial population per flask	Outcome of selection*				
	W2 (Indochina)	FCR3 ("The Gambia")	HB3 (Honduras)	3D7 ("Netherlands")	D6 (Sierra Leonne)
10^8	3 /3	3 /3	0 /3	3 /3	0 /3
10^7	3 /3	3 /3	0 /3	3 /3	0 /3
10^6	3 /3	3 /3	0 /3	3 /3	0 /3
10^5	3 /3	0 /3	0 /3	0 /3	0 /3
10^4	0 /3	0 /3	0 /3	0 /3	0 /3

Rathod *et al.* 1997

Only certain genetic backgrounds can develop ATO resistance

mtDNA Heteroplasmy

Next-generation sequencing reveals cryptic mtDNA diversity of *Plasmodium relictum* in the Hawaiian Islands

S. I. JARVI[1]*, M. E. FARIAS[1], D. A. LAPOINTE[2], M. BELCAID[3] *and* C. T. ATKINSON[2]

[1] *Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo, 34 Rainbow Drive, Hilo, HI 96720, USA*
[2] *U.S. Geological Survey, Pacific Island Ecosystems Research Center, Kilauea Field Station, P.O. Box 44, Building 343, Hawaii National Park, HI 96718, USA*
[3] *Information and Computer Sciences Department, University of Hawaii at Manoa, Pacific Ocean Science and Technology Building, Room 317, 1680 East-West Road, Honolulu, HI 96822, USA*

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PLoS NEGLECTED TROPICAL DISEASES

Multiple Mitochondrial Introgression Events and Heteroplasmy in *Trypanosoma cruzi* Revealed by Maxicircle MLST and Next Generation Sequencing

Louisa A. Messenger[1]*, Martin S. Llewellyn[1], Tapan Bhattacharyya[1], Oscar Franzén[2], Michael D. Lewis[1], Juan David Ramírez[3], Hernan J. Carrasco[4], Björn Andersson[2], Michael A. Miles[1]

1 Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, **2** Science for Life Laboratory, Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden, **3** Centro de Investigaciones en Microbiología y Parasitología Tropical, CIMPAT, Universidad de los Andes, Bogotá, Colombia, **4** Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela, Los Chaguaramos, Caracas, Venezuela

The Plant Cell, Vol. 10, 1163–1180, July 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

Stoichiometric Shifts in the Common Bean Mitochondrial Genome Leading to Male Sterility and Spontaneous Reversion to Fertility

Hanna Janska,[a,1] Rodrigo Sarria,[b,1] Magdalena Woloszynska,[a] Maria Arrieta-Montiel,[b] and Sally A. Mackenzie[b,2]

[a] Institute of Biochemistry and Molecular Biology, University of Wroclaw, Tamka, 2, 50-137 Wroclaw, Poland
[b] Department of Agronomy, Lilly Hall, Purdue University, West Lafayette, Indiana 47907

Research Article

SCI

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Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry

Hideo Ishii,[a]* James Fountaine,[a] Wen-Hsin Chung,[a] Masanori Kansako,[b] Kumiko Nishimura,[a] Kazuhito Takahashi[a,c] and Michiyo Oshima[a]

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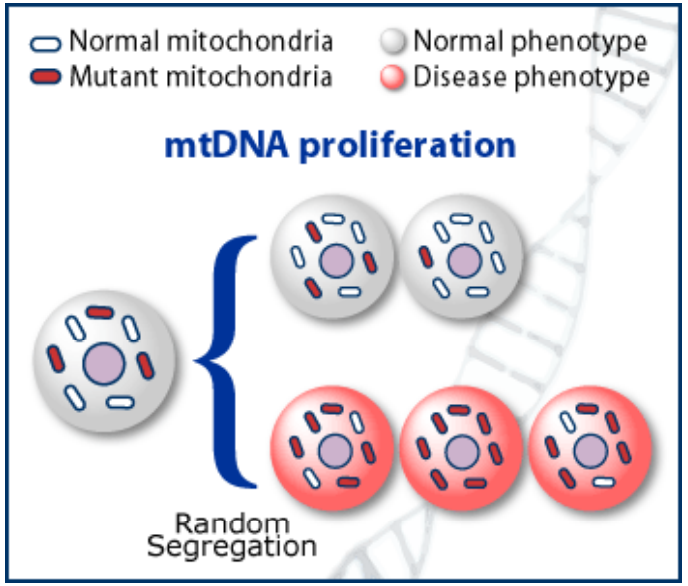
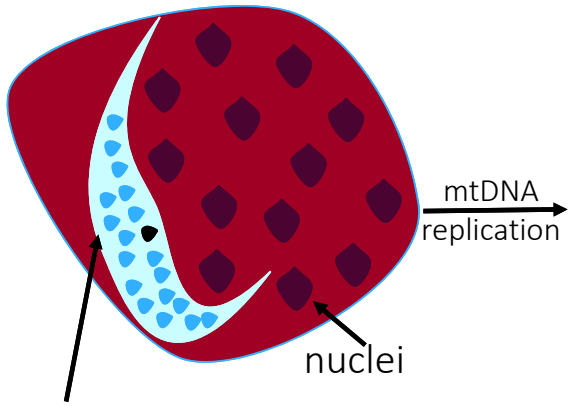
Heteroplasmy and homoplasmy

- Cells have multiple copies of the mt genome
- **Heteroplasmy** is the mixture of mtDNA copies with mutant and normal (WT) mtDNA in a single cell
- **Homoplasmy** refers to a cell that has a uniform collection of mtDNA, either completely normal (WT) or completely mutant
- During cell division, mtDNA replicates and sorts randomly among daughter cells



Schizont: nuclear replication

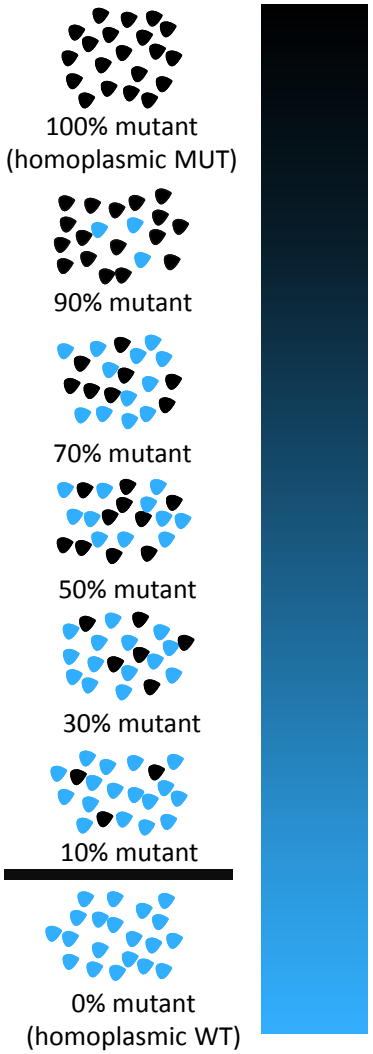


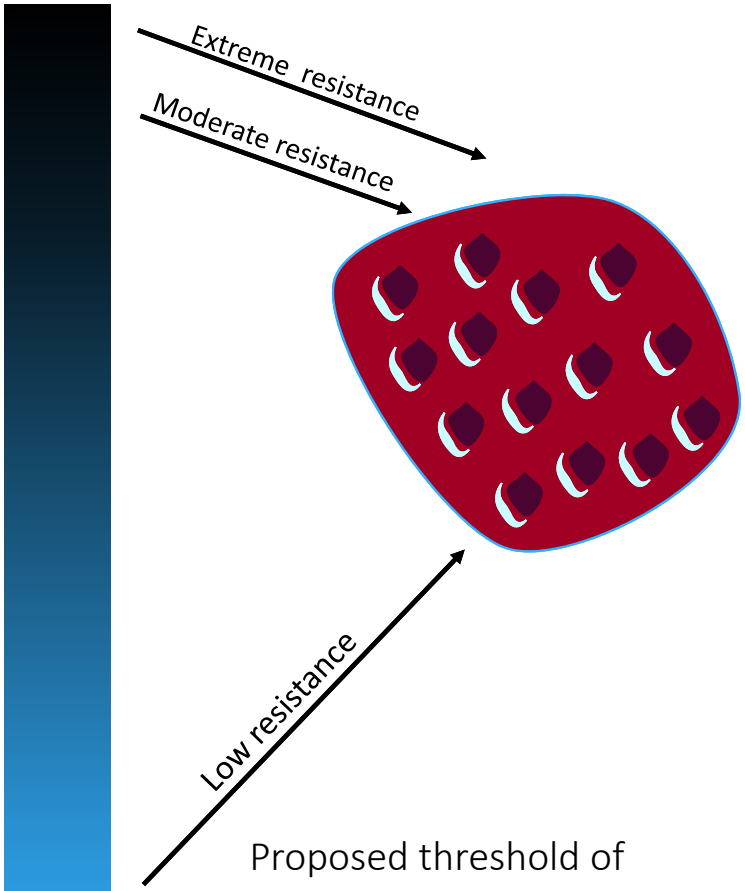
Mitochondria

- MUTANT mtDNA copy
- WT mtDNA copy

- Single elongated mitochondria
- ~22 copies of mtDNA
- maternally inherited

Schizont: mitochondrial replication possible outcomes

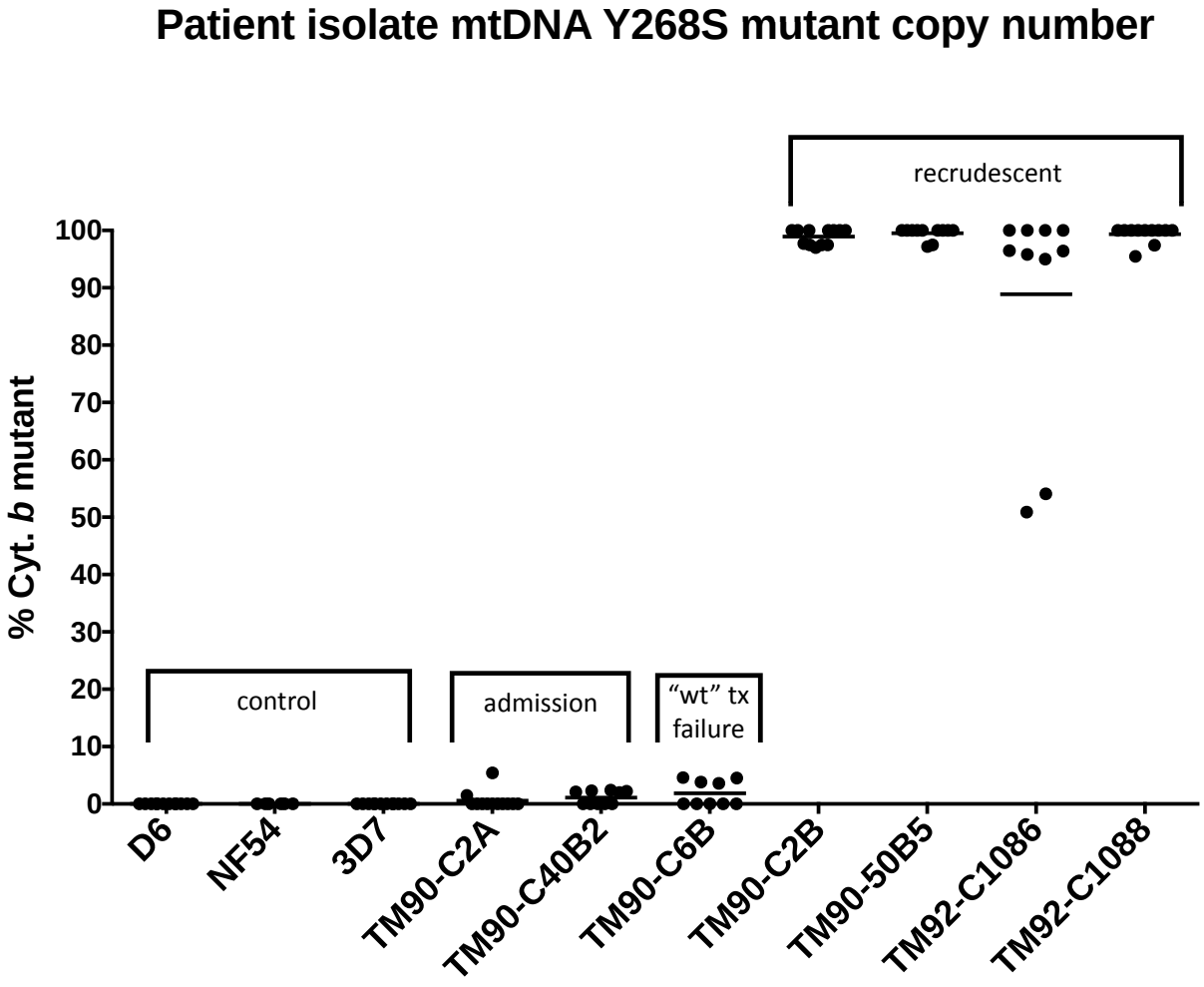




Proposed threshold of phenotypic resistance in heterogeneity of mtDNA

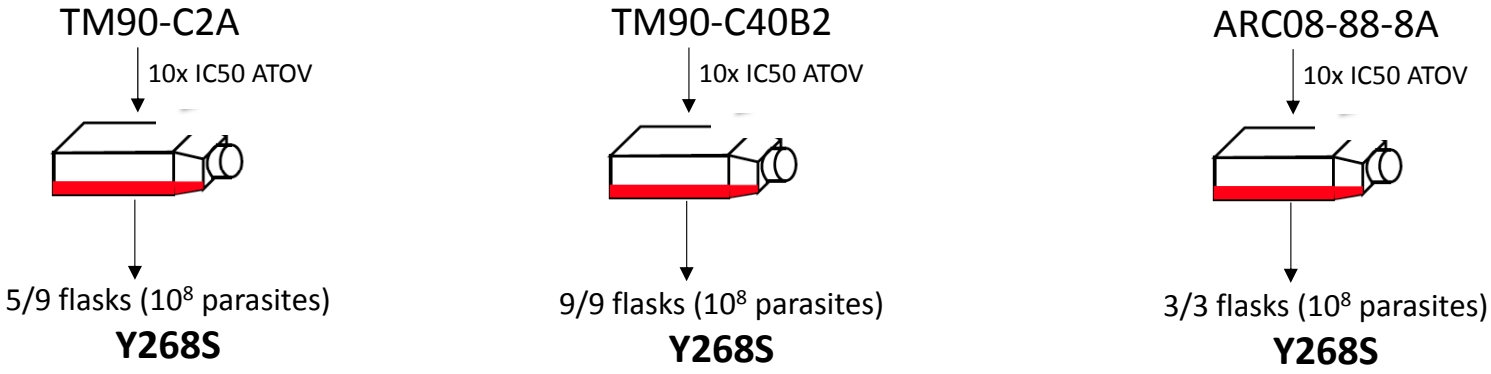
Resistance model of mtDNA heteroplasmy

Cryptic Y268 heteroplasmy detected in admission and recrudescent parasites with pyrosequencing



Siegel *et al.* 2018, BioRXIV

Using admission parasites, Y268S mutation is readily selected *in vitro*

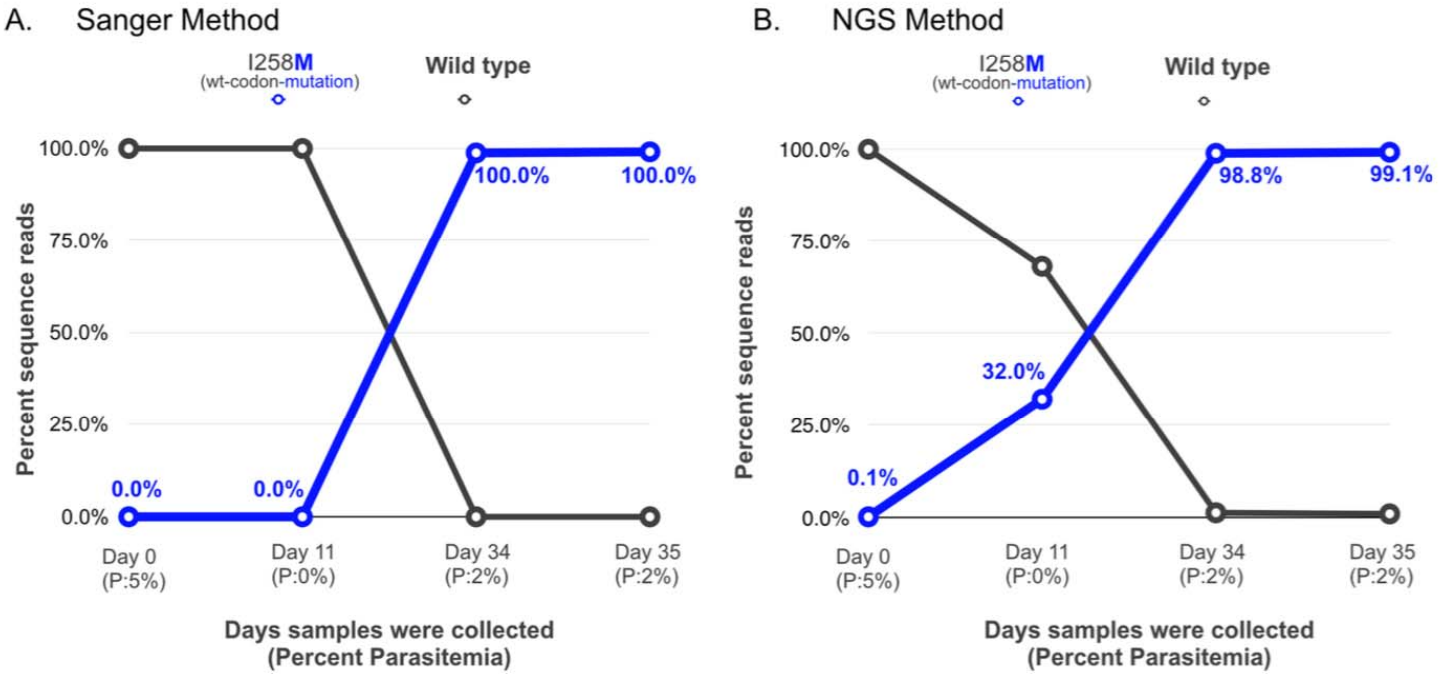


Initial resistance to mitochondrial electron transport chain inhibitors in atovaquone-selected populations of *P. falciparum* (EC_{50}, μM) and cytochrome *b* genotypes

Parasite	Qo site inhibitors		DHODH inhibitor	cyt. *b* genotype
	ATOV	MYX	DSM-1	
ARC08-88-8A	0.0076	ND	0.10	
TM90-C2A-F6	0.0013	0.094	0.12	--
TM90-C2B-A3	12	1.2	0.040	Y268S
C2A-F6+10x ATOV-1A	26.7	1.27	0.078	Y268S
C2A-F6+10x ATOV-3A	5.43	4.32	0.033	Y268S
C2A-F6+10x ATOV-1B	28.2	1.99	0.094	Y268S
C2A-F6+10x ATOV-2B	4.16	0.280	0.029	Y268S
C2A-F6+10x ATOV-3B	4.21	0.287	0.031	Y268S
ARC08-88-8A+10xATOV-1A	63	ND	0.067	Y268S

Siegel *et al.* 2018, BioRXIV

I258M mutation found prior to Malarone administration

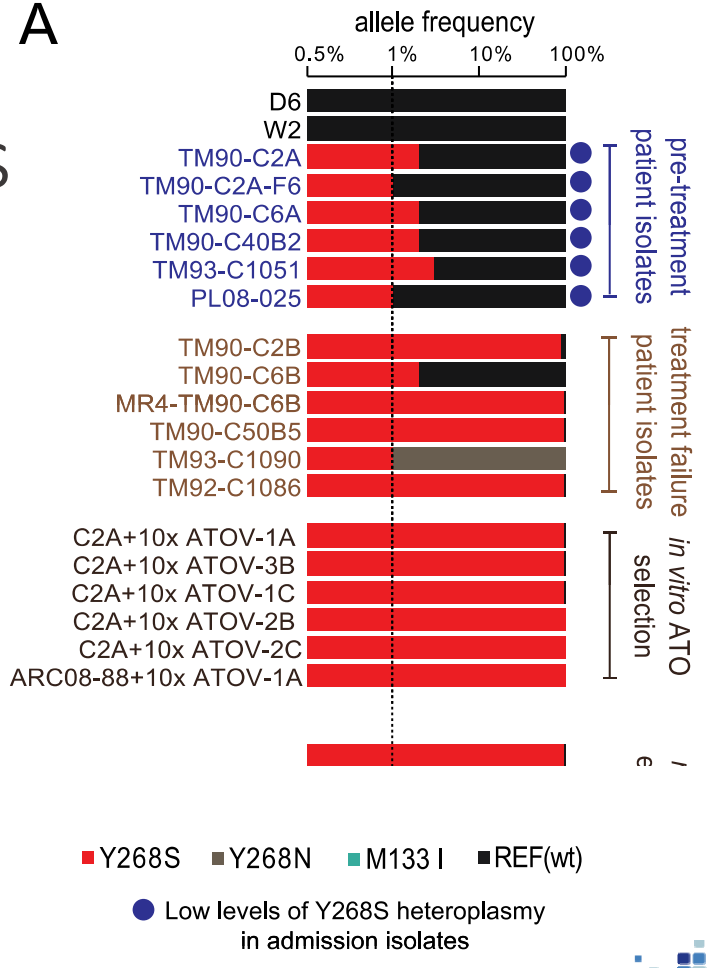


Talundzic *et al.* 2016

PCR-free Illumina sequencing detects heteroplasmic parasites

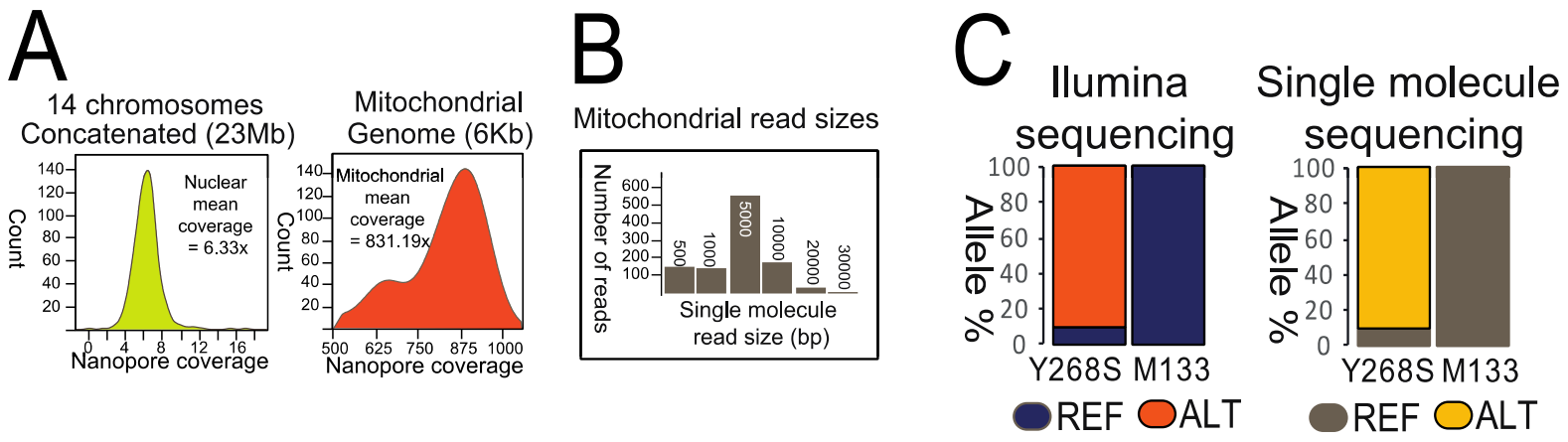
- Admission isolates have low-level Y268S heteroplasmy
- Recrudescent isolates have high-level Y268S heteroplasmy
- ATOV *in vitro* selected lines maintain Y268S genotype

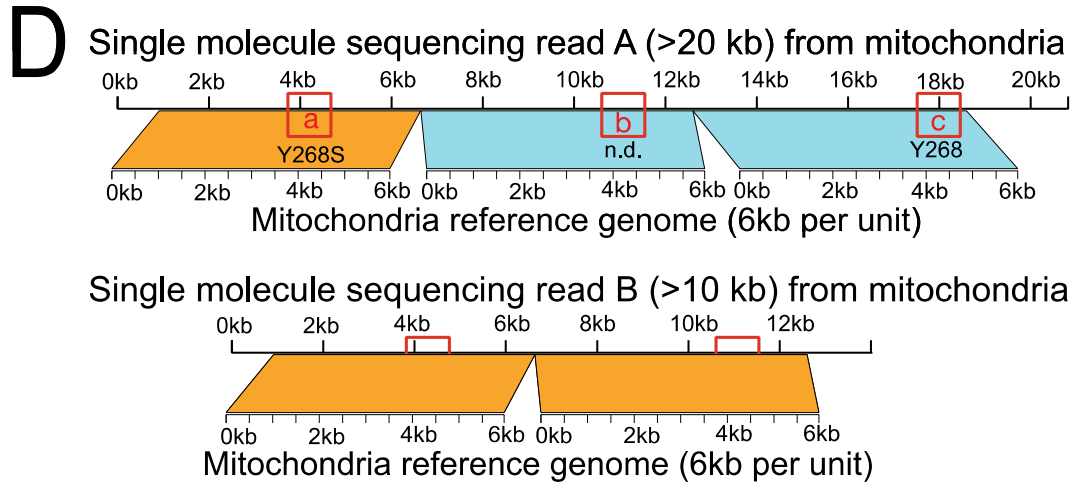
A



Siegel *et al.* 2018, BioRXIV

Single molecule sequencing detects heteroplasmic mtDNA



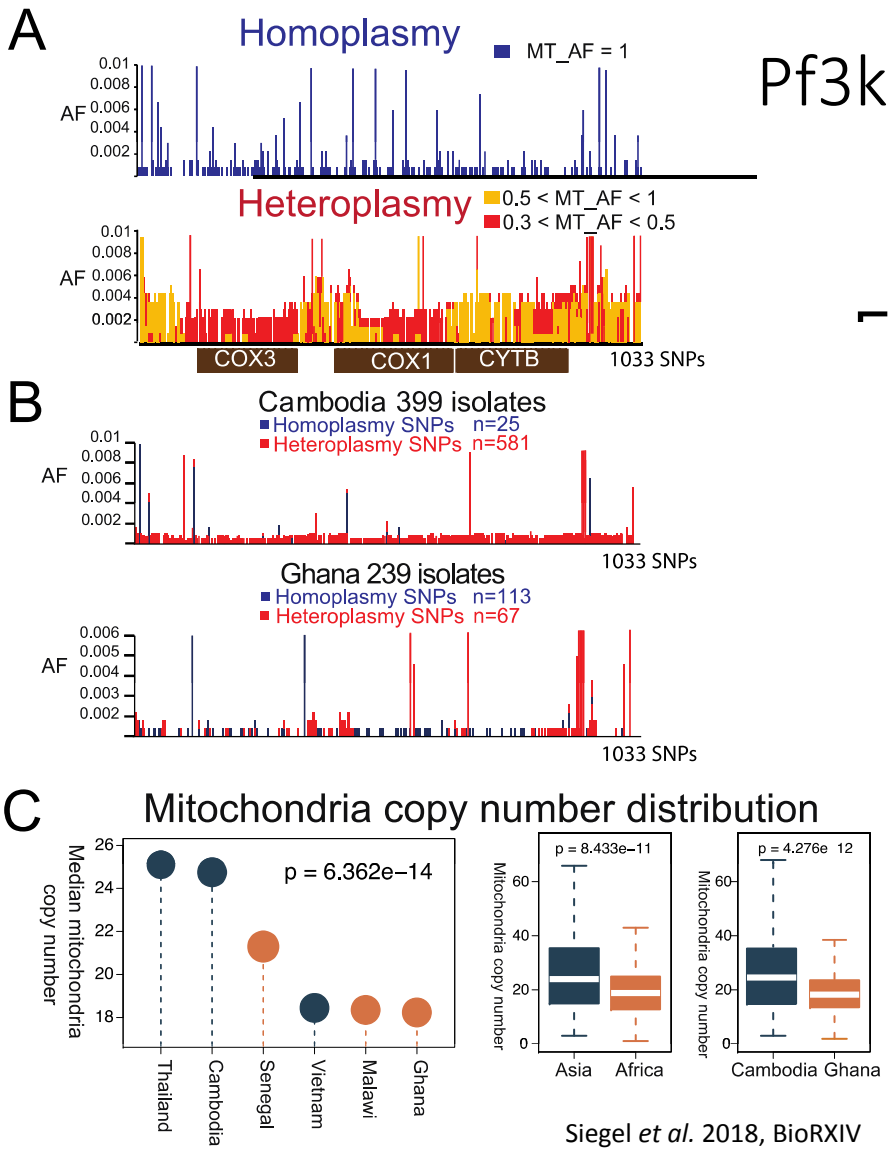


E

Reference	CAT	TTT	TAT	GCA	ATG
	H	F	Y	A	M
Read A Section a	CAT	TTT	TCT	GCA	ATG
	H	F	S	A	M
Read A Section b	-AT	TTT	-AT	-A	ATG
		F			M
Read A Section c	CAT	TTT	TAT	GCA	ATG
	H	F	Y	A	M
Section d	CAT	TTT	TCT	GCA	ATG
	H	F	S	A	M
Read B Section e	CAT	TTT	TCT	GCA	ATG
	H	F	S	A	M

Siegel *et al.* 2018, BioRXIV

Pf3k: global analysis of mtDNA diversity



Siegel *et al.* 2018, BioRXIV

- Heteroplasmy is a generator of mtDNA diversity
- Geographically distinct heteroplasmic haplotypes exist
- mtDNA copy number varies geographically

Conclusions

- First successful *in vitro* selection of the atovaquone Y268S mutation
- Pre-existing mitochondrial heteroplasmy explains the sporadic treatment failures with atovaquone
- Mitochondrial diversity is much higher than previously thought when heteroplasmic alleles are taken into account
- Mitochondrial copy number and heteroplasmy could have important implications for drug resistance mechanisms

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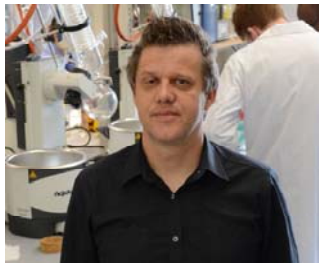

Roman Manetsch
Northeastern University

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Julian Rayner

Lia Chappell

Roberto Amato

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Kristin Lane
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