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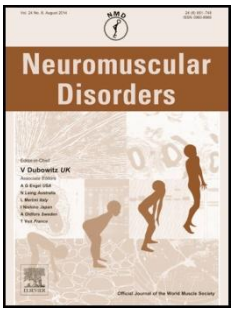

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Title: The role of p62/SQSTM1 in sporadic inclusion body myositis

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Highlights

- Two major deposits in s-IBM, p62 and LC3, join to perform selective autophagy.
- A variety of Lys63-ubiquitinated proteins can be accumulated with p62.
- Binding of p62-damaged proteins to LC3 could be incomplete in s-IBM muscle.
- Degradation of protein aggregates stops during autophagy before lysosome participation.

Abstract

We examined selective autophagy against ubiquitinated protein aggregates in sporadic inclusion body myositis (s-IBM) patients. The form of autophagy requires phosphorylation of serine 403 in p62/SQSTM1 to bind to Lys63-linked ubiquitin and the binding of the p62-ubiquitinated protein conjugates to LC3. In muscle biopsy specimens from 16 s-IBM patients, we compared the distribution of p62 (aa120-440) with 1) Ser403-phosphorylated p62 (S403-pp62), 2) Lys63-linked ubiquitin and 3) LC3 in double-colour immunofluorescence microscopy. S403-pp62, Lys63-linked ubiquitin and LC3 colocalised with p62 aggregates, 79.05% $\pm$ 13.64% (mean $\pm$ SD), 66.54% $\pm$ 19.91% and 51.84% $\pm$ 14.1%, respectively. Although positive deposits of S403-pp62 and Lys63-linked ubiquitin were always observed within p62

aggregates, LC3 often showed dissociated distribution from p62. We also found fibres containing small, numerous p62-positive dots that were negative for all three markers and were also observed in myositis controls. The results indicate that p62, Lys63-linked ubiquitin and LC3 in s-IBM join to perform selective autophagy. p62 could be induced by some cellular stresses in all types of myositis; however, in s-IBM, compromised binding of the p62-ubiquitinated protein complex to LC3 could stop the autophagy process in its initial stages, which causes the formation of aggregates of p62-oligomers with Lys63-ubiquitinated proteins.

Key words

sporadic inclusion body myositis (s-IBM), p62/SQSTM1, Lys63-linked ubiquitin, LC3, selective autophagy

1. Introduction

Sporadic inclusion body myositis (s-IBM) belongs to the family of idiopathic autoimmune inflammatory myopathies and is the most frequently acquired myopathy in individuals over 50 years of age. Unlike

polymyositis/dermatomyositis, it is refractory to immunotherapies. Its muscle pathology in routine histochemistry is characterised by the presence of rimmed vacuoles and mononuclear cells surrounding and invading non-necrotic muscle fibres. The mechanism of the disease remains to be determined [1,2].

Several investigations have indicated an abnormal expression of proteins associated with autophagy and lysosomes in muscle fibres with s-IBM [3-6]. Autophagy (more strictly, macroautophagy) is the bulk degradation of small cytoplasmic parts and organelles in nonselective manner. These are engulfed by double membrane-bound autophagosomes, which subsequently fuse with lysosomes. This process was originally described in cells undergoing starvation and has been well characterised [7]. Among the many proteins in this cycle, microtubule-associated protein 1A/1B-light chain 3 (LC3) plays a pivotal role from the early phase to the late phase of autophagy and it can be a good marker of autophagosomes [8]. A previous study showed that LC3 is a good histological marker of s-IBM [9]. On the other hand, aggregates of p62/SQSTM1 are constantly observed and are useful in the diagnosis of s-IBM [10-12]. p62 by itself is a multifunctional

protein that regulates various signal transduction pathways involved in cell survival and death [13]. It is a scaffold protein and has several binding sequences that can be recognised by other signal molecules (PB1, ZZ, TB, LIR and UBA domains) (Figure 1A). For example, through PB1, p62 interacts with itself, which facilitates to form self-oligomers. PB1 attracts various important signalling kinases, such as aPKC and ERK. ZZ is a zinc finger domain. TB binds to the pro-inflammatory signal protein TRAF6 [13].

Several studies have clearly shown that two domains of p62, LIR (LC3-interacting region) and UBA (ubiquitin-associated domain), are involved in selective autophagy. Although autophagy is generally thought to be nonselective degradation system, recent studies indicate that it is selective under some conditions, such as in the processing of ubiquitinated protein aggregates, damaged mitochondria, and intracellular pathogens [14]. p62 plays a significant role in autophagy of ubiquitinated protein aggregates, transporting them to the autophagy-lysosome degradation system (Figure 1B). During this process, phosphorylation of serine 403 on UBA of p62 increases the affinity of p62 to ubiquitin and autophagic clearance of the ubiquitinated proteins [15].

In s-IBM, many species of protein deposits have been detected in degenerative muscle fibres in addition to those of autophagy [11], Some studies have shown ubiquitin inclusions in s-IBM, although proteins that are ubiquitinated remain unknown [11]. It is reasonable to presume that the classical ubiquitin-proteasome pathway degrades abnormal proteins; however, proteasomes cannot process protein aggregates [16]. Selective autophagy functions by clearing aggregates of proteins and other molecules as well as organelles. When ubiquitin attaches to target molecules, chain elongation occurs at several lysine residues. Ubiquitin is added via Lys48 (Lys48-linked ubiquitin chains) in the ubiquitin-proteasome pathway, whereas Lys63-linked ubiquitin is preferentially used in selective autophagy [15,17]. Accordingly, neurons from p62-/- mice show the accumulation of Lys63-ubiquitinated proteins [18]. The molecular complex of ubiquitinated protein aggregated with p62 subsequently assembles with LC3 at the LIR domain. A mutation in the LIR domain resulted in the dissociated accumulation of p62 from LC3 in an experimental study. [19]

To determine whether p62 in s-IBM is involved in selective autophagy of ubiquitinated protein aggregates, we examined the

localisations of serine 403 phosphorylated p62 (S403-pp62), Lys63-linked ubiquitin and LC3 compared with that of p62. p62 was detected using antibodies against its backbone sequence aa120-440.

2. Materials and methods

2.1. Patients

We studied muscle biopsy specimens from patients with s-IBM, who fulfilled the clinicopathologically defined criteria of the s-IBM given by Dr. Rose and the ENMC IBM Working Group [20]. Table 1 shows the age, sex and disease duration of each patient. Polymyositis (n = 6), dermatomyositis (n = 4), necrotising myopathy (n = 3) and myofibrillar myopathies (n = 4) served as disease controls. Specimens with no or minor pathological alterations were used as normal controls (n = 4). Most of the biopsies in s-IBM and control patients were done from limb muscles (Table 1 and Supplementary Table 1). Diagnosis of myofibrillar myopathies was based on characteristic trichrome staining, and immunohistochemical studies that showed ectopic accumulation of various proteins including desmin and myotilin in abnormal regions [21]. One patient had a mutation in the *DES* gene. No mutations

were determined in the other patients.

Muscle biopsies were used after informed consent was received from all patients, and all procedures were approved by the ethics committees of the respective institutions.

2.2. Immunohistochemistry

Table 2 lists the antibodies used in the present study. Muscle biopsy specimens were frozen in isopentane cooled in liquid nitrogen and stored at -80°C. Cryostat sections were cut, fixed in cold acetone and blocked for 30 min with phosphate-buffered saline (PBS) containing 2 % bovine serum albumin (BSA) and 10% normal goat or horse serum. After blocking, the sections were incubated overnight at 4°C with the primary antibody against p62 (Medical & Biological Laboratories Co., Nagoya, Japan) diluted with the blocking solution. The sections were then incubated with a biotin-labelled secondary antibody and developed using the avidin–biotin complex immunoperoxidase method (Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine as the colouring agent. The control experiments involved the omission of the primary antibody or the substitution of the

primary antibody with non-immune rabbit or mouse IgG.

For the double-colour immunofluorescence studies, the sections were blocked with PBS containing 2% BSA and 10 % normal donkey serum. After blocking, the sections were incubated overnight at 4°C with anti-p62 plus anti-S403-pp62, anti-Lys63-linked ubiquitin, or anti-LC3, followed by incubation with donkey secondary antibodies for multiple fluorescence. After washing, the slides were mounted with Vectashield (Vector) and examined using an Olympus BX43 microscope (Tokyo, Japan) equipped for multiple fluorescence study. CellSens (Olympus) software was used for analysis. The anti-S403-pp62 antibody has been well characterized [15]. Mouse and rat monoclonal antibodies were detected using a FITC-coupled secondary antibody, while in rabbit polyclonal and monoclonal antibodies a Cy3-conjugated secondary antibody was used.

For the control study in the double-colour fluorescence study, we tested the specificity using anti-p62 antibody plus either normal rat, rabbit or mouse IgG (Santa Cruz Co., Santa Cruz, CA, USA) in place of a first antibody. We confirmed that there was no cross-reaction of p62 at the fluorescence intensity and filters used. Other control studies included a

single-colour fluorescence study using each first antibody and the omission of the primary antibody.

2.3. Statistics

All values are expressed as the mean ± standard deviation. Differences among means were analysed using one-way analysis of variance (ANOVA). When ANOVA showed significant differences, pair-wise comparisons were performed using Bonferroni's post-hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1, Localisation of p62 in immunoperoxidase method

In patients with s-IBM, a proportion of the fibres contained one to several aggregates of p62 (Figure 2, Table 1). These fibres were often smaller than usual and abnormal in shape. The aggregates were round, comma-shaped or irregular, and up to ~10 μm in the longitudinal axis. They were frequently associated with vacuoles. In addition, a proportion of the fibres harboured many small deposits of p62 (p62 puncta), which were up to about 0.8 μm in

diameter and evenly distributed. Fibres with p62 aggregates often contained p62 puncta deeper in the cytoplasm. When compared using hematoxylin and eosin (H&E) staining, in s-IBM, some fibres with p62 puncta seemed normal, while others were small or thick and round. However, they were not necrotic or regenerating. Fibres invaded by mononuclear cells were sometimes positive for p62 puncta. Positive fibres of p62 aggregates often displayed vacuoles and sometimes eosinophilic granules under H&E staining. In other types of myositis, a proportion of the fibres in some patients had p62 puncta, which were sometimes dense, but fibres with p62 aggregates (i.e., large immunoreactive products) were rarely found. In patients with myofibrillar myopathies, some of the fibres contained p62 aggregates. They appeared similar to those in s-IBM, but were sometimes larger and more irregularly shaped.

3.2. Double-colour fluorescence study

S403-pp62-positive products were usually detected identically to p62 aggregates (Figure 3A-3C). However, p62 puncta were often negative for S403-pp62. No S403-pp62-positive deposits were found outside the p62

deposits. Lys63-linked ubiquitin was often present with the p62 aggregates (Figure 3D-3F); no labelled deposits without p62 were found, although Lys63-linked ubiquitin was sometimes present diffusely in the cytoplasm and on sarcolemma. LC3 often colocalised with p62 aggregates, but discrepancies were also found (Figure 4). LC3-positive fibres were principally positive for p62.

For the calculations, we photographed, through a x40 objective lens, 10–15 pictures of fibres containing p62 aggregates in each marker of each case and examined whether p62 aggregates were positive for S403-pp62, Lys63-linked ubiquitin or LC3. When the two colour-labelled areas overlapped by over 75%, we classed it as G3; 50%–74% was classed as G2, 25–49% was classed as G1, and 0–24% was classed as G0. Table 3 shows the data of the numbers of fibres categorised into the different grades of overlap for each patient. G3 was the most frequent among the 4 grades in all the three markers. In the present paper, we defined the G3 level as colocalised.

S403-pp62, Ly63-linked ubiquitin and LC3 colocalised with p62 at means rates of 79.05% $\pm$13.64% (n = 16, mean $\pm$ SD), 66.54% $\pm$19.91% and 51.84% $\pm$14.1%, respectively. Statistically, the level of Ly63-linked ubiquitin

was not different from that of S403-pp62. However, the level of LC3 was significantly lower than that of S403-pp62 ($p < 0.0001$).

In other types of myositis, p62 puncta were virtually negative for S403-pp62 (Figure 5A), Lys63-linked ubiquitin and LC3 (Supplementary Table1). Overall, markers other than p62 were rarely found in the muscle cytoplasm in the myositis controls. In contrast to s-IBM, p62 aggregates in myofibrillar myopathies were partially positive for S403-pp62 even in large aggregates (Figure 5B). However, they contained some aggregates of Lys63-linked ubiquitin and LC3 with or without p62.

4. Discussion

In intact cells, protein aggregates are attached by Lys63-linked ubiqiuitin chains. p62 connects with these ubiquitin chains on UBA and next LC3 binds to the LC3-binding domain of p62 to proceed the autophagy-lysosome pathway (Figure 1). Phosphorylation at Ser403 of p62 increases the p62-binding affinity to ubiquitin [15]. In the present study, we demonstrated that p62 aggregated in the muscles of patients with s-IBM is largely phosphorylated at Ser403 and that Lys63-linked ubiqiuitin colocalised well

with p62 aggregates. The results indicate the abnormal emergence of the initiation process of selective autophagy against ubiquitinated proteins.

In s-IBM and some of the myositis controls, p62 puncta lacked S403-pp62 immunoreactivity. In contrast, p62 aggregates (i.e., large deposits) were detected almost exclusively in s-IBM and a high proportion of p62 in the aggregates is phosphorylated at Ser403. It is therefore possible that p62 puncta are induced by stresses, such as oxidative stress equally in s-IBM and in other types of myositis unrelated to autophagy. Experimentally, p62 is spotted in speckles, with the formation of the p62 self-oligomer by the sequence of the PB1 domain. p62 further forms speckles and binds with other signalling molecules, such as aPKC and TRAF6 [13]. p62 puncta that were not positive for S403-pp62 in this study could be such p62-related cytoplasmic speckles. A colocalisation study of p62 puncta with aPKC or TRAF6 will determine this hypothesis.

Histological studies have indicated the presence of a number of proteins in degenerative muscle fibres in s-IBM [11]. These proteins could be bound by Lys63-linked ubiquitin chains combined with p62 based on the results from the present study. Molecules modified by Lys63-ubiquitin chains

include those involved in DNA repair, kinase activation, or intracellular trafficking [22, 23]. Interestingly, our group found DNA damage and repair proteins in vacuolated or other abnormal fibres in s-IBM [24]. It is also intriguing that amyloid precursor protein (APP) is degraded by Lys63-linked polyubiquitination [25]; APP has been found in s-IBM muscle fibres [26, 11]. We suspect that defective selective autophagy cannot degrade p62 speckles that are induced by cellular stresses, which brings on the accumulation of various molecules modified by Lys63-ubiquitin chains. In selective autophagy of ubiquitinated protein aggregates, the p62-polyubiquitin-aggregated protein complexes bind to LC3 on the LIR domain of p62 and are incorporated into autophagosomes, which subsequently fuse to lysosomes (autolysosomes) (see Figure1). Protein aggregates are normally degraded inside autolysosomes. The comparison of p62 localisation with LC3 in the present study showed that LC3 is detected in fibres with p62 aggregates, and about one half of positive fibres showed a close coexistence of LC3 with p62. This ratio suggests that the selective autophagy progresses to the binding of p62 to LC3 on some level. At the same time, the rate of colocalisation, which was significantly lower in p62 vs. LC3

than in S403-pp62, permits the hypothesis that the autophagy pathway could lack accuracy, with incomplete binding of p62 to LC3. Such dissociated accumulation of p62 from LC3 has been observed in cultured cells with a mutation in the LIR domain of p62 [19]. Muscle fibres of s-IBM could lack accuracy in p62 biding to LC3, which results in holding the course of selective autophagy and bringing abnormal emergence of p62, Lys63-linked ubiquitin and LC3. On the other hand, LC3 not associated with p62 could be induced in the demand of classical autophagy itself, such as by nutrition deficit-like conditions. Also in this case, the remaining of LC3 could result from deficient autophagy.

In *VCP* mutations, which cause inclusion body myopathy, Paget's disease of bone and frontotemporal dementia (IBMPFD), the muscle pathology shows similar degeneration of s-IBM [27], revealing muscle fibres with rimmed vacuoles and tubulofilaments [28]. Immunohistochemistry shows aggregates of ubiquitin, p62, LC3 and TAR DNA-binding protein 43 (TDP-43) in experimental animals with IBMPFD mutant expression [29]. TDP-43 deposits have been observed in muscle fibres of s-IBM patients [30-32]. Moreover, VCP has been shown to be essential for the maturation of

ubiquitin-containing autophagosomes and this function is compromised by the presence of mutations [33]. It is therefore reasonable to consider that in s-IBM some molecules important for selective autophagy against ubiquitinated protein aggregates, particularly molecules for p62 binding to LC3, could be the target in the autoimmune process. It would be necessary to test the colocalisation of p62 with S403-pp62, Lys63-linked ubiquitin and LC3 in cases with *VCP* mutation to verify such a hypothesis.

Recent investigations have found that autoantibodies against cytosolic 5'-nucleotidase 1A are relevant to s-IBM [34, 35]. The family of cytosolic 5'-nucleotidases control changes in nucleoside and nucleotide concentrations [36]. In experiments, the inhibition of 5'-nucleotidase enzymes activates AMP-activated protein kinase (AMPK), which senses the shortage of ATP [37]. Activated AMPK turns on catabolic pathways to generate ATP and promotes autophagy [38]. Therefore, the autoantibody could be ultimately related to the induction of the aberrant selective autophagy.

In myofibrillar myopathies, various proteins, which are mainly present in the Z disc, are deposited within the muscle fibres [21]. This

accumulation could stimulate protein degradation systems. However, the present results showed that p62 is mostly not phosphorylated at serine 403; that is, the accumulation of proteins is not due to incomplete selective autophagy in the disease. Titin, the giant sarcomeric protein, has a kinase domain. By the activation of this kinase, p62 is targeted to sarcomeres by Nbr1 and involved in a signal transduction cascade to the nucleus upon muscle inactivity [39]. p62 could be accumulated with sarcomeric proteins under such circumstances.

In conclusion, we have provided evidence of deficient autophagy in s-IBM. We hypothesise that apparently complicated phenomena in s-IBM muscle pathology [1, 2] could be explained by an alteration of a single regulator of autophagy, as VCP in the IBMPFD muscle.

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Figure 1. Domains of the p62/SQSTM1 molecule and a schema of the autophagy-lysosome system that degrades ubiquitinated protein aggregates. 1) PB 1: the domain for self-oligomer formation, ZZ: ZZ-type zinc finger domain, TB: a TRAF6-binding domain, LIR: LC3-interacting region, UBA: ubiquitin-associated domain. Phosphorylation of Ser403 in this domain increases the affinity of p62 to ubiquitin. 2) Protein aggregates bounded by Lys63-ubiquitin form complexes with p62. They attached to LC3 and become incorporated to a phagophore, which grows into an autophagosome (a structure with double layer membranes). The autophagosome is combined with a lysosome to become an autolysosome, in which aggregates are finally degraded.

Figure 2. p62/SQSTM1 stained by the immunoperoxidase method using monoclonal antibody against aa120–440. (A) Several p62-positive aggregates are seen in a small fibre with abnormal contours (arrow). Note that some other fibres (arrow heads) contain numerous small dots of p62 deposits (p62 puncta). (B) Many p62 aggregates associate with vacuoles. Bar = 20 μm

Figure 3. Double-colour immunofluorescence study. p62 (A1, B1, C1: Cy3) and Serine 403-phosphorylated p62 (S403-pp62) (A2, B2, C2: FITC); p62 (D1,

E1, F1: FITC) and Lys63-ubiquitin (D2, E2, F2: Cy3). Aggregates of S403-pp62 colocalised with p62 (A, B, C). Note that p62-positive deposits in C1, less than 0.5–1 μm in diameter (p62 puncta) do not show positive results for S403-pp62 (C2). Aggregates of p62 are positive for Lys63-ubiquitin (D, E, F). p62 puncta inside the fibre are Lys63-ubiquitin negative (F2). Bar = 20 μm

Figure 4. Immunofluorescence. p62 (A1, B1, C1: Cy3) and LC3 (A2, B2, C2: FITC). LC3 colocalises p62 (A). However, in (B), LC3-immunoreactive products largely do not associate with p62 aggregates (e.g. arrows). (C) LC3 is present diffusely in the muscle fibre, while p62 forms aggregates. Bar = 20 μm

Figure 5. Immunofluorescence. (A) p62 puncta in a case of polymyositis. p62 (A1), S403-pp62 (A2). p62 puncta are negative for S403-pp62. (B) Myofibrillar myopathy. p62 (B1), S403-pp62 (B2). p62 aggregates are seen. However, only trace deposits of S403-pp62 are observed. Bar = 20 μm

Table and figure legends

Table 1. Profiles of the 16 patients with sporadic inclusion body myositis (s-IBM). The ratios of fibres with rimmed vacuoles and those positive for p62/SQSTM1 of each patient are shown in the last two columns. The percentages of fibres with p62 aggregates are always higher than those with rimmed vacuoles.

Patient no.	Age/sex	Biopsy site	Disease duration (years)	% of fibres with rimmed vacuoles	% of fibres with p62 aggregates
IBM-1	82/M	QF	1.3	6.21	7.52
2	60/M	BB	5	3.92	12.17
3	74/M	BB	6	2.66	5.26
4	56/M	QF	5	1.79	6.12
5	47/M	QF	1.3	0.62	2.16
6	75/M	BB	4	2.30	7.80
7	64/M	QF	1.2	0.88	5.11
8	69/M	QF	4	2.61	20.00
9	65/F	BB	3	0.83	5.06
10	66/M	BB	3	1.34	11.01
11	71/M	QF	1.4	2.83	9.09
12	50/M	TB	1.3	2.62	11.75
13	74/M	BB	10	2.56	5.43
14	81/M	BB	4	1.20	4.23
15	70/M	QF	5	3.57	4.51
16	77/F	BB	4	0.04	2.74

BB: biceps brachii, QF: quadriceps femoris, TR: triceps brachii.

Table 2. First and secondary antibodies used in the study. Abbreviations.

MAA: mouse monoclonal antibody, RPA: rabbit polyclonal antibody, RatMA: rat monoclonal antibody, RbMA: rabbit monoclonal antibody. MBL: Medical & Biological Laboratories

First antibodies				
Antigen	Antibody	Clone/ID	Supplier	Concentration/ dilution
p62/SQSTM1(aa 120–440)	MMA	5F2	MBL	1:200
p62/SQSTM1(aa 120–440)	RPA	PM045	MBL	1:500
Phospho-p62 / (SQSTM1) (Ser403)	RatMA	4F6	MBL	10 μg/mL
ubiquitin, Lys63-specific	RbMA	Apu3	Millipore	1:20
LC3	MMA	4E12	MBL	40 μg/mL
Secondary antibodies				
Antibody	Host animal	ID	Supplier	Concentration/ dilusion
Biotinylated anti-mouse IgG	Horse	BA-2000	Vector	1:200
Biotinylated anti-rabbit IgG	Goat	BA-1000	Vector	1:200
Anti-mouse IgG, FITC conjugate	Donkey	AP192F	Millipore	1:50
Anti-rabbit IgG, Cy3 conjugate	Donkey	AP182C	Millipore	1:200
Anti-rat IgG, FITC conjugate	Donkey	AP189P	Millipore	1:200

Table 3. Results of the double-colour immunofluorescence study. The number of fibres categorised into each grade of overlap is shown. G3: overlapped by >75%; G2: 50%–74%; G1: 25%–49%; G0: <24%. For example, on Ser403-phosphorylated p62 (S403-pp62) aggregates compared with p62 in patient 1, 8 fibres were classed as G3.

Patient No.	Serine 403 phosphorylated p62 vs. p62					Lysine 63 linked ubiquitin vs. p62					LC3 vs. p62				
	G3 (No. of fibres)	G2	G1	G0	G3/ Total (n) (%)	G3	G2	G1	G0	G3/ Total (n) (%)	G3	G2	G1	G0	G3/ Total (n) (%)
IBM-1	8	2	0	0	80.00	7	3	0	1	63.64	8	2	1	0	72.73
2	10	1	0	2	76.92	8	3	1	1	61.54	7	3	4	4	38.89
3	8	1	1	0	80.00	8	1	0	1	80.00	4	3	3	0	40.00
4	11	0	1	0	91.67	4	3	3	3	30.77	11	2	1	0	78.57
5	5	2	2	1	50.00	5	1	3	1	50.00	5	1	2	1	55.56
6	16	3	0	2	76.19	13	1	5	2	61.9	6	4	5	0	40.00
7	6	2	0	2	60.00	12	0	2	0	85.71	9	4	1	3	52.94
8	14	0	2	0	87.50	12	0	0	0	100	5	2	1	1	55.56
9	6	0	2	1	66.67	4	2	0	3	44.44	6	1	4	2	46.15
10	9	0	2	0	81.82	8	2	2	1	61.54	7	3	0	2	58.33
11	13	3	0	1	76.47	12	0	0	0	100	4	7	4	1	25.00
12	12	0	0	0	100	7	1	4	1	53.85	8	2	2	4	50.00
13	8	2	2	0	66.67	6	1	1	1	66.67	7	6	1	0	50.00

14	11	0	0	0	100	7	0	2	2	63.64	11	1	1	1	78.57
15	10	0	1	0	90.91	10	1	0	0	90.91	5	3	4	0	41.67
16	8	2	0	0	80.00	5	2	1	2	50.00	5	3	3	0	45.45
Total	155	18	13	9	79.05% ± 13.64%	128	21	24	19	66.54% ± 19.91%	108	47	37	19	51.84% ± 14.1%